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14. ABSTRACT

Ovarian cancer ranks first as the cause of death for gynecological cancers. Obviously, there is an urgent need to develop novel treatment methods for ovarian cancer. To do this, we must better understand key events associated with ovarian cancer development. In order to combat cancer, a normal cell's typical response to a tumor-promoting genetic alteration is irreversible growth arrest, consequently preventing the normal human cell from progressing towards becoming a cancer cell. This process is termed senescence. When this process fails, those cells containing tumor-promoting genetic alterations can grow without control and become a tumor. The potential use of cellular senescence for cancer therapy would rely on reactivation of this process in cancer cells. We have previously discovered a pathway that opposes the beneficial process of senescence. This pathway is referred to as the canonical Wnt signaling pathway. Therefore, we hypothesize that canonical Wnt signaling pathway contributes to the development of ovarian cancer through bypassing senescence. The proposed studies may lead to development of strategies for ovarian cancer treatment using reactivation of cellular senescence as a novel mechanism by targeting ovarian cancer promoting canonical Wnt signaling.

15. SUBJECT TERMS
Ovarian Cancer, Cellular senescence, Wnt signaling

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Introduction:

The role of Wnt signaling in epithelial ovarian cancer (EOC) development remains largely elusive. We have evidence to suggest that canonical Wnt signaling is activated through downregulation of Wnt5a in EOC. Wnt5a is a non-canonical Wntligand that acts as an antagonist of canonical Wnt signaling. Furthermore, inhibition of canonical Wnt signaling or restoration of Wnt5a expression inhibits the growth of EOC cells. The overall hypothesis of this proposal is that canonical Wnt signaling activated by loss of Wnt5a contributes to EOC development by abrogation of OIS. The objectives of the proposed studies are to determine the role of canonical Wnt signaling in regulating OIS of ovarian epithelial cells during progression of benign ovarian tumors into invasive EOCs, and to investigate the effects of inhibition of the canonical Wnt signaling on malignant behavior of EOC cells. The specific aims are: 1): Elucidate the mechanisms by which inhibition of canonical Wnt signaling inhibits the growth of EOC cells; 2): Determine whether loss of Wnt5a contributes to EOC development by abrogation of OIS; 3): Investigate the effects of inhibition of canonical Wnt signaling on malignant behavior of EOC cells in immunodeficient mice.

Body:

1. Research accomplishments associated with each task outlined in the approved Statement of Work.

Task 1. Determine whether inhibition of canonical Wnt signaling inhibits the growth of EOC cells through inducing cell senescence. (Months 1-12)

Specifically, we will determine whether inhibition of canonical Wnt signaling induces the expression of markers of senescence in human EOC cells.

1a) Determine the effect of inhibition of canonical Wnt signaling by a small molecule inhibitor FJ9 on expression of markers of senescence in human EOC cells. (Months 1-6).

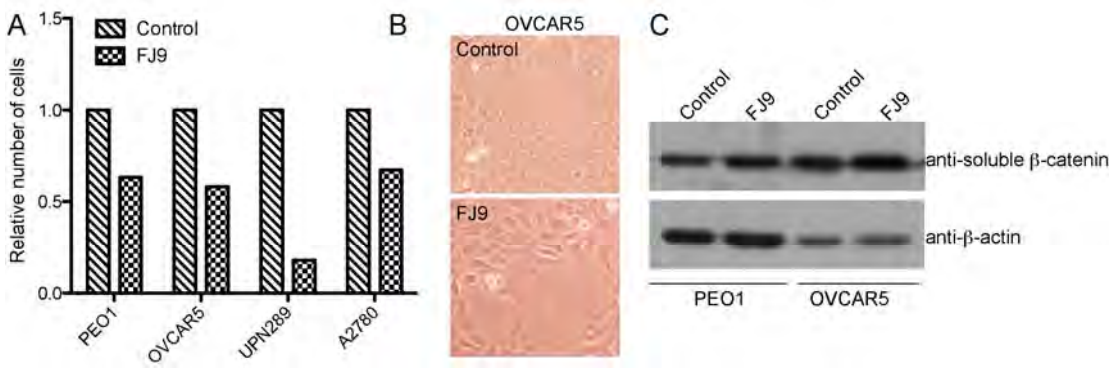

Figure 1. FJ9 suppresses the growth of human EOC cells via Wnt/β-catenin independent mechanisms. (A) Equal number of indicated EOC cell lines were treated with 100uM FJ9 or vehicle control (dissolved in 0.01% DMSO). After 72 hours of treatment, the number of cells were counted and calculated as relative number of cells. (B) same as (A) but examined for cell morphology changes using phase contrast microscopy. (C) Same as (A) but examined for expression of soluble b-catenin and b-actin in indicated human EOC cell lines treated with FJ9 or vehicle control by immunoblotting using indicated antibodies.

Progress report: FJ9 has previously been demonstrated as an inhibitor of canonical Wnt signaling [1]. We showed that FJ9 inhibits the growth of human EOC cells in a panel of human EOC cell lines (Figure 1A). Consistent with the idea that this is due to senescence induction, cells treated with FJ9 demonstrated features of senescence such as a large flat cell morphology (Figure 1B). However, examination of markers of canonical Wnt signaling in FJ9 treated or control cells showed that there is no evidence to suggest the observed effects are due to inhibition of canonical Wnt/b-catenin pathway. For example, the levels of soluble b-catenin, a marker of active canonical Wnt/b-catenin pathway were not decreased by FJ9 treatment. This result suggests that FJ9 inhibits the growth of human EOC cells via canonical Wnt signaling independent mechanisms.

1b) Determine the effect of inhibition of canonical Wnt signaling by restoration of Wnt5a expression on expression of marker of senescence in human EOC cells. (Months 7-12)

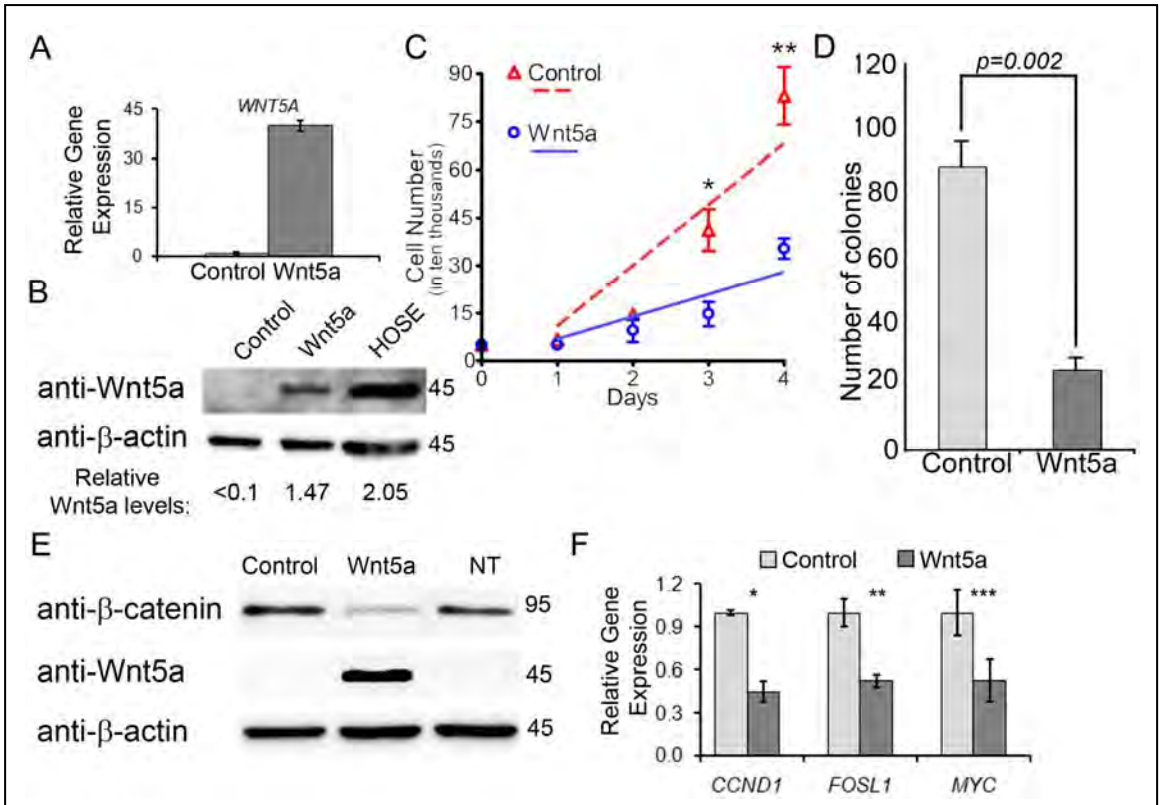

Figure 2. Wnt5a restoration inhibits the growth of human EOC cells by antagonizing canonical Wnt/β-catenin signaling. (A) OVCAR5 cells were transduced with a control or Wnt5a encoding puromycin resistant retrovirus. The infected cells were drug-selected with 3 μg/ml puromycin. Expression of Wnt5a mRNA in drug-selected cells was determined by qRT-PCR. **(B)** Same as (A), but examined for expression of Wnt5a and β-actin in control or Wnt5a-infected OVCAR5 and primary HOSE cells by immunoblotting. Relative levels of Wnt5a expression was indicated based on densitometric analysis using NIH Image J software. **(C)** Same as (A), but equal number (5000) of drug-selected control (open triangles and dotted line) or Wnt5a-infected cells (open circles and solide line) were cultured on plastic plates for 4 days, and the number of cells was counted (Control ± SD or Wnt5a ± SD (n=3); student *t-test* was used for calculating p value) *p = 0.0038 and **p = 0.001. Mean of three independent experiments with SD and linear regression. **(D)** Same as (C), but grown under anchorage-independent condition in soft-agar. The number of colonies was counted 2 weeks after initial inoculation. Mean of three independent experiments with SD. **(E)** Same as (A), but examined for the levels of soluble β-catenin and β-actin expression by immunoblotting (NT = non-treated). **(F)** Same as (A), but examined for expression of indicated β–catenin target genes by qRT-PCR. Expression of *β*-2-microglobulin was used to normalize the expression of indicated genes. * p = 0.0095, ** p = 0.0012 and *** p = 0.0286 compared with controls.

Progress report: We sought to determine the effects of Wnt5a reconstitution in human EOC cells. Wnt5a expression was reconstituted in the OVCAR5 EOC cell line via retroviral transduction. Ectopically expressed Wnt5a was confirmed by both qRT-PCR and immunoblotting in OVCAR5 cells stably expressing Wnt5a or a vector control

(Figure 2A-B). Of note, the levels of ectopically expressed Wnt5a in OVCAR5 cells are comparable to the levels observed in primary HOSE cells (Figure 2B). Interestingly, Wnt5a reconstitution in OVCAR5 human EOC cells significantly inhibited both anchorage-dependent and anchorage-independent growth in soft-agar compared with vector controls (Figure 2C-D). In addition, similar growth inhibition by Wnt5a reconstitution was also observed in the PEO1 human EOC cell line (Figure 3A-C), suggesting that this effect is not cell line specific. Based on these results, we conclude that Wnt5a reconstitution inhibits the growth of human EOC cells in vitro.

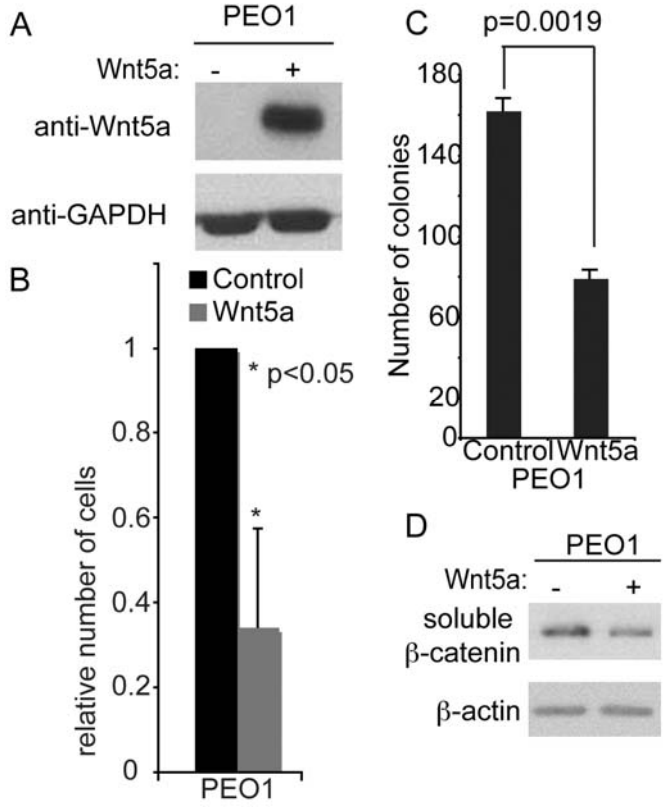

Figure 3. Wnt5a restoration inhibits the growth of PEO1 EOC cells by antagonizing canonical Wnt signaling. **(A)** PEO1 cells were transduced with a control- or Wnt5a-encoding puromycin resistant retrovirus. The infected cells were drug-selected with 1 μg/ml puromycin for 3 days. Expression of Wnt5a and GAPDH in drug-selected cells was determined by immunoblotting. **(B)** Same as (A) but an equal number of drug-selected control and Wnt5a infected PEO1 cells were cultured on plastic plate for 5 days and the number of cells were counted using trypan blue exclusion assay. Mean of three independent experiments with SD. **(C)** Same as (B) but grown under anchorage-independent conditions in soft-agar. The number of colonies were counted 2 weeks after initial inoculation. Mean of 3 independent experiments with SD. **(D)** Same as (A), but examined for soluble β-catenin expression by immunoblotting.

Canonical Wnt signaling promotes cell proliferation and Wnt5a has been demonstrated to antagonize the canonical Wnt/β-catenin signaling in certain cell contexts [2-5]. We hypothesized that Wnt5a would suppress the growth of human EOC cells by antagonizing canonical Wnt/β-catenin signaling. To test our hypothesis, we examined the effect of Wnt5a reconstitution on expression of markers of active Wnt/β-catenin signaling in human EOC cells, namely the levels of "active" soluble β-catenin[6-8] and expression of β-catenin target genes such as CCND1, c-MYC and FOSL1 [9, 10]. Indeed, we observed a decrease in soluble β-catenin in Wnt5a reconstituted OVCAR5 cells compared with vector controls (Figure 2E). Consistently, we also observed a significant decrease in the levels of β-catenin target genes in these cells, namely CCND1

(p = 0.0095), FOSL1 (p = 0.0012) and c-MYC (p = 0.0286) (Figure 2F). Similar effects of Wnt5a reconstitution on expression of markers of active Wnt/β-catenin signaling (such as decreased levels of solubleβ-catenin) were also observed in PEO1 human EOC cells (Figure 3), suggesting that this is not cell line specific. Based on these results, we conclude that Wnt5a suppresses the growth of human EOC cells by antagonizing canonical Wnt/β-catenin signaling in human EOC cells.

Next, we sought to determine the cellular mechanism whereby Wnt5a inhibits the growth of human EOC cells. We have previously shown that suppression of canonical Wnt signaling promotes cellular senescence in primary human fibroblasts by activating the senescence-promoting histone repressor A (HIRA)/ promyelocyticleukemia (PML) pathway [8]. PML bodies are 20-30 dot-like structures in the nucleus of virtually all human cells. PML bodies are sites of poorly defined tumor suppressor activity, and are disrupted in acute promyelocytic leukemia [11]. PML has been implicated in regulating cellular senescence. For example, the foci number and size of PML bodies increase during senescence [11, 12] and inactivation of PML suppresses senescence [13]. Activation of the HIRA/PML pathway is reflected by the recruitment of HIRA into PML bodies [14].

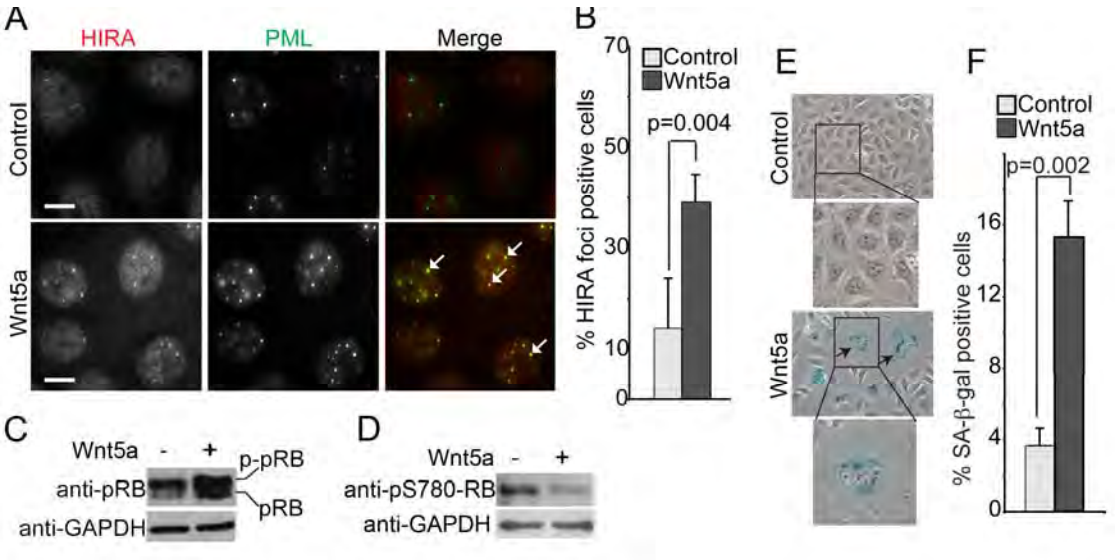

Figure 4. Wnt5a restoration triggers cellular senescence in human EOC cells. (A) Control and Wnt5a expressing OVCAR5 EOC cells were stained with antibodies to HIRA and PML. Arrows point to examples of co-localized HIRA and PML bodies. Bar = 10 μm. **(B)** Quantitation of (A). 200 cells from control and Wnt5a expressing cells were examined for HIRA and PML co-localization. Mean of three independent experiments with SD.**(C)** Same as (A) , but examined for pRB and GAPDH expression. (**D**) Same as (C), but examined for pRBpS780 and GAPDH expression. **(E)** Same as (A), but examined for senescence-associated β-galactosidase (SA-β-gal) activity. **(F)** Quantitation of (E). Mean of three independent experiments with SD.

As Wnt5a antagonizes canonical Wnt signaling in human EOC cells (Figure 2E-F), we sought to determine whether Wnt5a restoration might activate the senescence-promoting HIRA/PML pathway and induce senescence in human EOC cells. Towards this goal, we examined the localization of HIRA in OVCAR5 EOC cells reconstituted

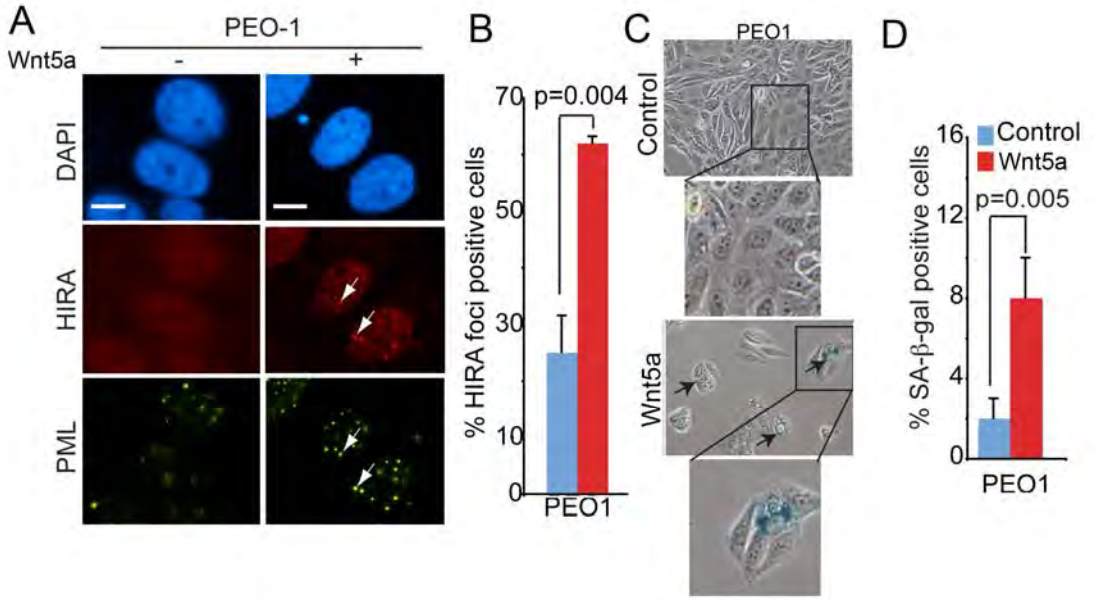

Figure 5. Wnt5a restoration triggers senescence in human EOC cells. (A) PEO1 EOC cells were infected with control or Wnt5a encoding puromycin-resistant retrovirus. The infected cells were drug-selected using 1 mg/ml of puromycin. Drug-selected cells were stained with antibodies to HIRA and PML. Arrows point to examples of co-localized HIRA and PML bodies. Bar = 10 mm. **(B)** Quantitation of (A). Mean of three independent experiments with SD. **(C)** Same as (A) but stained for SA-b-gal activity. **(D)** Quantitation of (C). Mean of three independent experiments with SD.

with Wnt5a or vector control. Notably, there was a significant increase in the percentage of cells with HIRA localized to PML bodies in Wnt5a restored human EOC cells compared with controls (Figure 4A-B, $p = 0.004$). In addition, we also observed an increase in the number and size of PML bodies in the Wnt5a restored OVCAR5 EOC cells (Figure 4A), which are also established markers of cellular senescence [13, 15].

The p53 and pRB tumor suppressor pathways play a key role in regulating senescence [16]. Thus, we sought to determine whether activation of the HIRA/PML pathway depends upon the p53 and pRB pathways. Interestingly, $p16^{INK4a}$, the upstream repressor of pRB, is deleted in OVCAR5 human EOC cell line [17]. In addition, the levels of total phosphorylated pRB were not decreased by Wnt5a, while the levels of cyclin D1/CKD4-mediated Serine 780 phosphorylation on pRB (pRBpS780) were decreased by Wnt5a [18] (Figure 4C-D). Further, p53 is null in OVCAR5 cells [19]. We conclude that activation of the HIRA/PML pathway is independent of the p53 and $p16^{INK4a}$.

Similarly, we observed activation of the HIRA/PML pathway by Wnt5a restoration in PEO1 human EOC cells (Figure 5A-B), suggesting that the observed effects are not cell line specific. Together, we conclude that Wnt5a reconstitution activates the HIRA/PML senescence pathway.

We next sought to determine whether Wnt5a restoration induces SA-β-gal activity, a universal marker of cellular senescence [16]. Indeed, SA-β-gal activity was notably induced by Wnt5a reconstitution in both OVCAR5 and PEO1 human EOC cells compared with controls (Figure 4E-F and 5C-D, respectively). Based on these results, we concluded that Wnt5a restoration induced senescence of human EOC cells by activating the HIRA/PML senescence pathway.

Key Research Accomplishments:

- FJ9 suppresses the growth of human EOC cells but likely through canonical Wntsignaling independent mechanisms.
- Wnt5a suppresses the growth of human EOC cells.
- Wnt5a inhibits canonical Wnt signaling.
- Wnt5a induces cellular senescence.

Reportable Outcomes:

Manuscripts:

1. Bitler BG, Nicodemus JP, Li H, Cai Q, Wu H, Hua X, Li T, Birrer MJ, Godwin AK, Cairns P, Zhang R. Wnt5a Suppresses Epithelial Ovarian Cancer by Promoting Cellular Senescence.Cancer Res. 2011 Oct 1;71(19):6184-94. Epub 2011 Aug 4.

2. Li H, Cai Q, Godwin AK, Zhang R. Enhancer of zeste homolog 2 promotes the proliferation and invasion of epithelial ovarian cancer cells.Mol Cancer Res. 2010 Dec;8(12):1610-8.

Abstracts:

An abstract based on this study has been published by American Association of Cancer Research.

Presentations:

A poster has been presented at 2011 AACR annual meeting.

Patents:

A patent has been filed based on the funded studies.
U.S. Patent Application Serial Number: 61/445, 145.

Conclusions:

Wnt5a promotes senescence of human EOC cells by suppressing the proliferation promoting canonical Wnt/β-catenin pathway. We suggest that strategies to drive senescence in EOC cells by reconstituting Wnt5a signaling may offer an effective new strategy for EOC therapy

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Appendices:

Manuscripts:

1. Bitler BG, Nicodemus JP, Li H, Cai Q, Wu H, Hua X, Li T, Birrer MJ, Godwin AK, Cairns P, Zhang R. Wnt5a Suppresses Epithelial Ovarian Cancer by Promoting Cellular Senescence.Cancer Res. 2011 Oct 1;71(19):6184-94. Epub 2011 Aug 4.

2. Li H, Cai Q, Godwin AK, Zhang R. Enhancer of zeste homolog 2 promotes the proliferation and invasion of epithelial ovarian cancer cells.Mol Cancer Res. 2010 Dec;8(12):1610-8.

Abstracts:

An abstract based on this study has been published by American Association of Cancer Research.

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Cancer Research

Wnt5a Suppresses Epithelial Ovarian Cancer by Promoting Cellular Senescence

Benjamin G. Bitler, Jasmine P. Nicodemus, Hua Li, et al.

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Wnt5a Suppresses Epithelial Ovarian Cancer by Promoting Cellular Senescence

Benjamin G. Bitler[1], Jasmine P. Nicodemus[1], Hua Li[1], Qi Cai[2], Hong Wu[3], Xiang Hua[4], Tianyu Li[5], Michael J. Birrer[7], Andrew K. Godwin[8], Paul Cairns[6], and Rugang Zhang[1]

Abstract

Epithelial ovarian cancer (EOC) remains the most lethal gynecologic malignancy in the United States. Thus, there is an urgent need to develop novel therapeutics for this disease. Cellular senescence is an important tumor suppression mechanism that has recently been suggested as a novel mechanism to target for developing cancer therapeutics. Wnt5a is a noncanonical Wnt ligand that plays a context-dependent role in human cancers. Here, we investigate the role of Wnt5a in regulating senescence of EOC cells. We show that Wnt5a is expressed at significantly lower levels in human EOC cell lines and in primary human EOCs ($n = 130$) compared with either normal ovarian surface epithelium ($n = 31$; $P = 0.039$) or fallopian tube epithelium ($n = 28$; $P < 0.001$). Notably, a lower level of Wnt5a expression correlates with tumor stage ($P = 0.003$) and predicts shorter overall survival in EOC patients ($P = 0.003$). Significantly, restoration of Wnt5a expression inhibits the proliferation of human EOC cells both *in vitro* and *in vivo* in an orthotopic EOC mouse model. Mechanistically, Wnt5a antagonizes canonical Wnt/β-catenin signaling and induces cellular senescence by activating the histone repressor A/promyelocytic leukemia senescence pathway. In summary, we show that loss of Wnt5a predicts poor outcome in EOC patients and Wnt5a suppresses the growth of EOC cells by triggering cellular senescence. We suggest that strategies to drive senescence in EOC cells by reconstituting Wnt5a signaling may offer an effective new strategy for EOC therapy. *Cancer Res; 71(19); 6184–94. ©2011 AACR.*

Introduction

Cellular senescence is an important tumor suppression mechanism *in vivo* (1). In primary mammalian cells, cellular senescence can be triggered by various inducers including critically shortened telomeres and activated oncogenes (such as oncogenic *RAS*; ref. 1). Senescent cells are viable but nondividing (2). Senescent cells also exhibit several distinctive morphologic characteristics and molecular markers, including a large flat cellular morphology and expression of senescence-associated β-galactosidase (SA-β-gal) activity (3). In murine liver carcinoma and sarcoma models, reactivation of the tumor suppressor p53 induces senescence and is associated with tumor regression (4, 5). Hence, driving cancer cells to undergo cellular senescence represents a novel mechanism for developing cancer therapeutics (6, 7).

More than 85% of ovarian cancers are of epithelial origin (8). Epithelial ovarian cancers (EOC) are classified into distinct histologic types including serous, mucinous, endometrioid, and clear cell (9). The most common histology of EOC is serous (~60% of all cancers) and less common histologies include endometrioid, clear cell, and mucinous (9). Recently, an alternative classification has been proposed, in which EOC is broadly divided into 2 types (10). Type I EOC includes endometrioid, mucinous, low-grade serous, and clear-cell carcinomas, and type II EOC includes high-grade serous carcinomas (10). EOC remains the most lethal gynecologic malignancy in the United States (8). Thus, there is an urgent need to better understand the etiology of EOC to develop novel therapeutics for this devastating disease.

Wnt signaling is initiated by binding of the Wnt ligand to its cognate frizzled receptor (11). Canonical Wnt signaling results in stabilization of the key transcription factor β-catenin, which then translocates into the nucleus and drives expression of its target genes, such as *CCND1 (cyclin D1), FOSL1,* and *c-MYC* (12, 13). Canonical Wnt signaling is active in the putative somatic stem/progenitor cells of the coelomic epithelium of the mouse ovary (14). Underscoring the importance of Wnt signaling in EOC, in a murine ovarian cancer model, activation of canonical Wnt signaling cooperates with inactivation of the tumor suppressor PTEN in driving ovarian carcinogenesis (15). However, the role of Wnt signaling in EOC is not fully understood.

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Wnt5a is a noncanonical Wnt ligand that plays opposing roles in different types of cancer and has variable expression dependent on the cancer context (16). Specifically, in EOC the role of Wnt5a remains unclear. Thus, in this study, we investigated Wnt5a expression and its potential function in human EOC cells. We discovered that Wnt5a was expressed at significantly lower levels in primary human EOC compared with either primary human ovarian surface epithelium or fallopian tube epithelium. Notably, loss of Wnt5a expression was associated with tumor stage and predicted shorter overall survival in EOC patients. Significantly, Wnt5a reconstitution inhibited the growth of EOC cells both *in vitro* and *in vivo* in an orthotopic EOC mouse model by promoting cellular senescence. These studies show, for the first time, a functional role of the noncanonical Wnt ligand, Wnt5a, in promoting senescence. Importantly, they also suggest that promoting EOC cells to undergo senescence represents a potential novel strategy for developing urgently needed EOC therapeutics.

Materials and Methods

Cells and culture conditions

Primary human ovarian surface epithelial (HOSE) cells were isolated and cultured as previously described (17). Human EOC cell lines were obtained from American Type Culture Collection (ATCC) and were passaged for less than 6 months. EOC cell line identification was further confirmed by DNA Diagnostic Center (www.dnacenter.com). EOC cell lines were cultured according to ATCC in RPMI-1640 medium supplemented with 10% FBS. 5-Aza-cytidine (Aza-C; Sigma) was used at working concentration of 5 μmol/L (18).

Human ovarian specimens and immunohistochemistry

The protocol to evaluate deidentified human tissue specimens was approved by Fox Chase Cancer Center (FCCC) institutional review board. Ovarian tumor microarray and normal human ovary and fallopian tube specimens were obtained from the FCCC Biosample Repository Core Facility (BRCF). Histopathology of the selected specimens on the tumor microarrays was provided by BRCF. Immunohistochemistry (IHC) was conducted by using goat anti-Wnt5a polyclonal antibody (R&D Systems) and mouse anti–Ki-67 (Dako) with a DAKO EnVision System and the Peroxidase (DAB) kit (DAKO Corporation) following the manufacturer's instructions and as previously described (17). Wnt5a staining was scored in a double-blinded manner by Dr. Qi Cai at the BRCF, and a proportion of the cases were independently confirmed by Dr. Hong Wu, a board-certified pathologist, at the FCCC Department of Pathology.

Anchorage-independent soft agar colony formation assay

Soft agar assay were carried out as previously described (17). Briefly, 3,500 cells were resuspended in 0.35% low melt agarose dissolved in RPMI-1640 medium supplemented with 10% FBS and inoculated on top of 0.6% low melt agarose base in 6-well plates. After 2 weeks in culture, the plates were stained with 0.005% crystal violet, and the number of colonies was counted by using a dissecting microscope.

Retrovirus production, infection, and drug selection

The following retrovirus constructs were used: pBABE-puro was obtained from Addgene, hygro-pWZL-luciferase was a kind gift of Dr. Denise Connolly, and pBABE-Wnt5a was generated by using standard cloning protocol. Retrovirus packaging was done as previously described by using Phoenix packaging cells (19, 20). To increase infection efficacy, double virus infection was carried out. For drug selection, 3 μg/mL of puromycin was used for the OVCAR5 human EOC cell line.

Reverse transcriptase PCR, quantitative reverse transcriptase PCR, and immunoblotting

RNA from cultured primary HOSE cells or human EOC cell lines was isolated by using TRIzol (Invitrogen) according to manufacturer's instruction. For quantitative reverse transcriptase PCR (qRT-PCR), TRIzol-isolated RNA was further purified by using an RNeasy kit (QIAGEN) following manufacture's instruction. The *Wnt5a*, *CCND1, FOSL1,* and *c-MYC* primers used for qRT-PCR were purchased from SABiosciences. mRNA expression of the housekeeping gene β-2-microglobulin (B2M) was used to normalize mRNA expression. Soluble β-catenin was extracted, using a buffer that consisting of 10 mmol/L Tris-HCl (pH 7.5), 0.05% NP-40, 10 mmol/L NaCl, 3 mmol/L $MgCl_2$, 1 mmol/L EDTA, and protease inhibitors (Roche) as previously described (21, 22). The following antibodies were used for immunoblotting from the indicated suppliers, goat anti-Wnt5a (R&D Systems), mouse β-actin (Sigma), mouse anti-GAPDH (Millipore), mouse anti-β-catenin, mouse anti-Rb (BD Biosciences), and rabbit anti-pRBpS780 (Cell Signaling).

Immunofluorescence and SA-β-gal staining

Indirect immunofluorescence staining was carried out as previously described (19, 20, 22). The following antibodies were used for immunofluorescence: a cocktail of mouse anti-HIRA monoclonal antibodies (WC19, WC117, and WC119; 1:10; ref. 20) and a rabbit anti-PML antibody (Chemicon, 1:5,000). Images were captured by a DS-QilMc camera on a Nikon Eclipse 80i microscope and processed by NIS-Elements BR3.0 software (Nikon). SA-β-gal staining was carried out as described previously (3, 23). For SA-β-gal staining in sections from xenografted tumors, 8 separate fields were examined from 2 individual tumors for each of the groups.

In vivo orthotopic xenograft tumorigenesis study

The protocol was approved by the FCCC Institutional Animal Care and Use Committee. OVCAR5 cells were infected with a luciferase-encoding retrovirus (hygro-pWZL-luciferase) and infected cells were selected with 50 μg/mL hygromycin. Drug-selected cells were then infected with control or Wnt5a-encoding retrovirus and subsequently selected with 3 μg/mL puromycin and 50 μg/mL hygromycin. A total of 3×10^6 drug-selected cells were unilaterally injected into the ovarian bursa sac of immunocompromised mice (6 mice per group; ref. 24). From day 10 postinfection, tumors were visualized by injecting luciferin (intraperitoneal, 4 mg/mice) resuspended in PBS and

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imaged with an IVIS Spectrum imaging system every 5 days until day 30. Images were analyzed by Live Imaging 4.0 software. At day 30, tumors were surgically dissected and either fixed in 10% formalin or fresh-frozen in Optimal Cutting Temperature compound (Tissue-Tek). Sections of the dissected tumors were processed by the FCCC Histopathology Core Facility.

Statistical analysis

Quantitative data are expressed as mean ± SD, unless otherwise indicated. ANOVA with Student's t test was used to identify significant differences in multiple comparisons. The Pearson χ^2 test was used to analyze the relationship between categorical variables. Overall survival was defined as the time elapsed from the date of diagnosis and the date of death from any cause or the date of last follow-up. Kaplan–Meier survival plots were generated and comparisons were made by using the log-rank sum statistic. For all statistical analyses, the level of significance was set at 0.05.

Results

Wnt5a is expressed at significantly lower levels in human EOC cell lines and primary human EOCs compared with normal human ovarian surface epithelium or fallopian tube epithelium

To determine Wnt5a expression in human EOC cell lines and primary HOSE cells, we examined the relative Wnt5a mRNA levels by carrying out semiquantitative RT-PCR. We observed that Wnt5a mRNA levels were greatly diminished in human EOC cell lines compared with primary HOSE cells (Fig. 1A). This finding was further confirmed through qRT-PCR analysis of Wnt5a mRNA in multiple isolations of primary HOSE cells and human EOC cell lines, showing that the levels of Wnt5a mRNA were significantly lower in human EOC cell lines compared with primary HOSE cells (Fig. 1B; $P = 0.008$). Consistently, we observed that Wnt5a protein levels were also lower in human EOC cell lines compared with primary HOSE cells as determined by immunoblotting (Fig. 1C). On the basis of these results, we conclude that Wnt5a is expressed at lower levels in human EOC cell lines compared with primary HOSE cells.

We next determined whether the loss of Wnt5a expression found in human EOC cell lines was also observed in primary human EOCs. We examined Wnt5a expression in 130 cases of primary human EOC specimens and 31 cases of normal human ovary with surface epithelium by IHC, using an antibody against Wnt5a (Table 1). In addition, there is recent evidence to suggest that a proportion of high-grade serous EOC may arise from distant fallopian tube epithelium (25). Thus, we also included 28 cases of normal human fallopian tube specimens in our IHC analysis (Table 1). The specificity of the anti-Wnt5a antibody was confirmed in our study (Supplementary Fig. S1). A single band at predicted molecular weight (~42 kDa) was detected in OVCAR5 cells with ectopically expressed Wnt5a and was absent after expression of a short hairpin RNA to the human Wnt5a gene (shWnt5a), which effectively knocked down Wnt5a mRNA expression (Supplementary Fig. S1A and data not shown). In addition, Wnt5a staining was lost when primary anti-Wnt5a antibody was replaced with an isotype-matched IgG control (Supplementary Fig. S1B).

As shown in Figure 1D, in normal human ovarian surface epithelial cells and fallopian tube epithelial cells, both cytoplasm and cell membrane were positive for Wnt5a IHC staining (black arrows, Fig. 1D). In contrast, Wnt5a staining in EOC cells was dramatically decreased (Fig. 1D). We scored expression of Wnt5a as high (H-score ≥ 30) or low (H score < 30) on the basis of a histological score (H score; 26), which considers both intensity of staining and percentage of positively stained cells, as previously described (17). Wnt5a expression was scored as high in 58.1% (18/31) cases of normal human ovarian surface epithelium and 82.1% (23/28) cases of normal human fallopian tube epithelium (Table 1). In contrast, Wnt5a expression was scored as high in 37.7% (49/130) cases of primary human EOCs (Table 1). Statistical analysis revealed that Wnt5a was expressed at significantly lower levels in primary human EOCs compared with either normal human ovarian surface epithelium ($P = 0.039$) or normal human fallopian tube epithelium ($P < 0.001$; Table 1). On the basis of these studies, we conclude that Wnt5a is expressed at significantly lower levels in primary human EOCs compared with either normal human ovarian surface epithelium or fallopian tube epithelium.

Wnt5a expression negatively correlates with tumor stage and lower Wnt5a expression predicts shorter overall survival

We next examined the correlation between Wnt5a expression and clinical and pathologic features of human EOCs. Significantly, there was a negative correlation between Wnt5a expression and tumor stage ($P = 0.003$; Table 1). Notably, the majority of examined cases are high-grade serous subtypes that are usually of stage 3/4. In addition, we examined the correlation between expression of Wnt5a and a marker of cell proliferation, Ki-67 (ref. 27; Fig. 1E). There was a significant negative correlation between Wnt5a expression and Ki-67 ($P = 0.038$; Table 1). We next assessed whether Wnt5a expression based on H score might predict prognosis of EOC patients (High, H score ≥ 30; Low, H score < 30; $n = 123$), for which long-term follow-up data were available. Significantly, lower Wnt5a expression correlated with shorter overall survival in the examined EOC patients ($P = 0.003$; Fig. 1F). Together, we conclude that a lower level of Wnt5a expression correlates with tumor stage and predicts shorter overall survival in human EOC patients.

Wnt5a gene promoter hypermethylation contributes to its downregulation in human EOC cells

Wnt5a gene promoter hypermethylation has been implicated as a mechanism underlying its silencing in several types of human cancers (16). Consistently, we also observed *Wnt5a* gene promoter hypermethylation in a number of human EOC cell lines (Fig. 2A; Supplementary Table S1). Further supporting a role of promoter hypermethylation in suppression of Wnt5a expression, treatment with a DNA demethylation drug, Aza-C (28), in PEO1 EOC cells resulted in a significant increase in levels of both Wnt5a mRNA and protein (Fig. 2B and C). We

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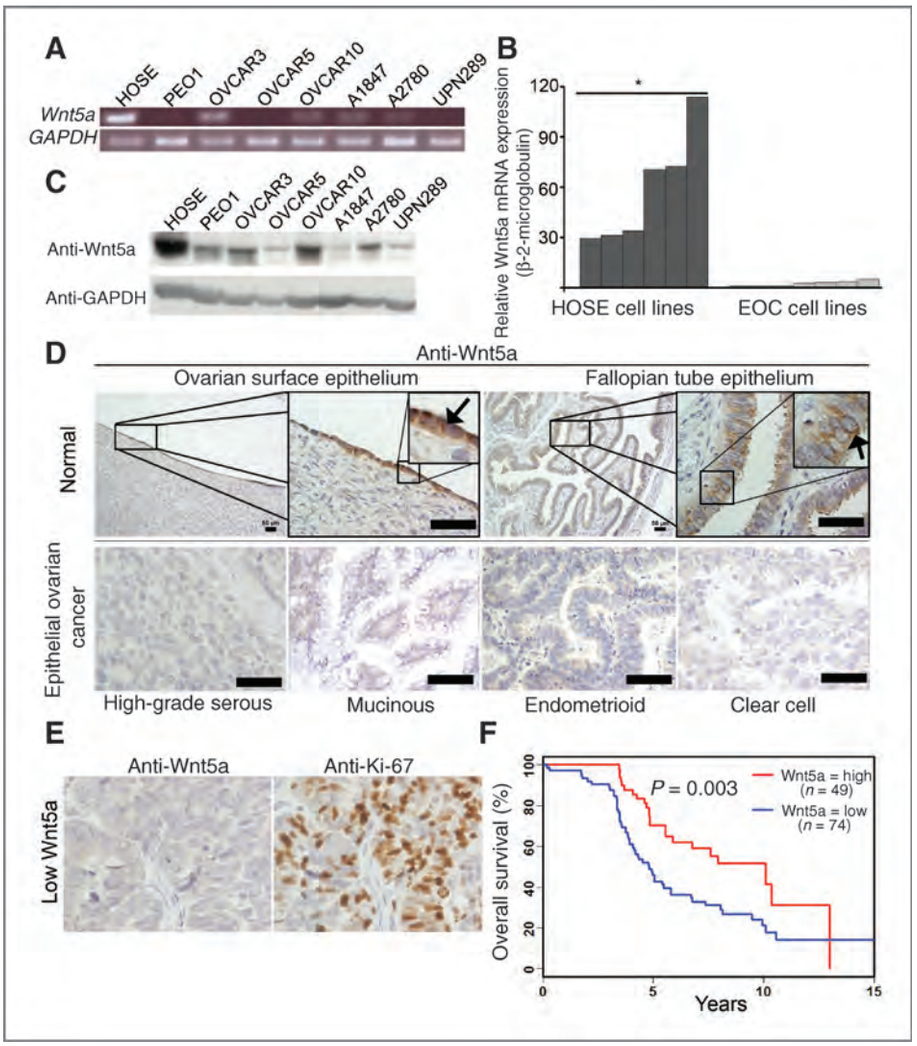

Figure 1. Wnt5a is expressed at significantly lower levels in human EOC cells compared with normal human ovarian surface or fallopian tube epithelial cells, and a lower level of Wnt5a expression predicts shorter overall survival in human EOC patients. A, expression of Wnt5a mRNA in primary HOSE cells and the indicated human EOC cell lines was determined by semiquantitative RT-PCR. Expression of glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) mRNA was used as a loading control. B, Wnt5a mRNA levels were quantified by qRT-PCR in 6 individual isolations of primary HOSE cells and 7 different EOC cell lines. Expression of β-2-microglobulin was used to normalize Wnt5a mRNA expression. *, $P = 0.008$ compared with human EOC cells. C, same as (A), but examined for Wnt5a and GAPDH protein expression by immunoblotting. D, examples of Wnt5a IHC staining in normal human ovarian surface epithelium, fallopian tube epithelium, and EOC of indicated histologic subtypes. Bar, 50 μm. Arrows point to examples of positively stained human ovarian surface epithelial cells and fallopian tube epithelial cells. E, representative images from tissue microarray depicting low Wnt5a expression correlated with high Ki-67, a cell proliferation marker. F, loss of Wnt5a expression is an independent poor prognosis marker in human EOC patients. A lower level of Wnt5a expression correlates with shorter overall survival in human EOC patients. The univariate overall survival curve (Kaplan–Meier method) for EOC patients ($n = 123$) with high- or low-Wnt5a expression as determined by immunohistochemical analysis.

conclude that *Wnt5a* gene promoter hypermethylation contributes to its downregulation in human EOC cells.

Wnt5a restoration inhibits the growth of human EOC cells by antagonizing the canonical Wnt/β-catenin signaling

We next sought to determine the effects of Wnt5a reconstitution in human EOC cells. Wnt5a expression was reconstituted in the OVCAR5 EOC cell line via retroviral transduction. Ectopically expressed Wnt5a was confirmed by both qRT-PCR and immunoblotting in OVCAR5 cells stably expressing Wnt5a or a vector control (Fig. 3A and B). Of note, the levels of ectopically expressed Wnt5a in OVCAR5 cells are comparable with the levels observed in primary HOSE cells (Fig. 3B). Interestingly, Wnt5a reconstitution in OVCAR5 human EOC cells significantly inhibited both anchorage-dependent and anchorage-independent growth in soft agar compared with vector controls (Fig. 3C and D). In addition, similar growth inhibition by Wnt5a reconstitution was also observed in the PEO1 human EOC cell line

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Table 1. Wnt5a expression in primary human EOCs and correlation of its expression with clinicopathologic variables

Patient characteristics	Wnt5a protein expression				
	Low (*n*)	High (*n*)	Total (*n*)	High (%)	*P*
Age (23–85 y; mean 59.2 y)					
≤55	24	16	40	40.0	
>55	52	33	85	38.8	0.900
Unknown	5	0	5		
Laterality					
Left	22	14	36	38.9	
Right	12	9	21	42.9	0.957
Bilaterality	35	24	59	40.7	
Undetermined	12	2	14		
Histotype					
EOC	81	49	130	37.7	
Type I	16	21	37	56.8	
Low-grade serous	1	1	2	50.0	
Endometrioid	4	9	13	69.2	
Mucinous	2	3	5	60.0	
Clear cell	5	4	9	44.4	
Others	4	4	8	50.0	
Type II					
High-grade serous	65	28	93	30.1	0.005[a]
Normal epithelium					
Ovarian surface	13	18	31	58.1	0.039[b]
Fallopian tube	5	23	28	82.1	<0.001[b]
Ki-67					
Low	22	23	44	52.3	
High	51	24	75	32.0	0.038
Undetermined	7	3	11		
Tumor grade					
1	3	7	10	70.0	
2	12	8	20	40.0	
3	64	31	95	32.6	
Undetermined	2	3	5		
Tumor stage					
Stage 1/2	12	18	30	60.0	
Stage 3/4	67	29	96	30.2	0.003[c]
Undetermined	2	2	4		

[a]Compared with type I EOC.
[b]Compared with EOC.
[c]Compared with stage 1/2.

(Supplementary Fig. S2A–C) suggesting that this effect is not cell line specific. On the basis of these results, we conclude that Wnt5a reconstitution inhibits the growth of human EOC cells *in vitro*.

Canonical Wnt signaling promotes cell proliferation and Wnt5a has been shown to antagonize the canonical Wnt/β-catenin signaling in certain cell contexts (16, 29–31). Because Wnt5a expression inversely correlated with expression of Ki-67 (Fig. 1E; Table 1), a cell proliferation marker, we hypothesized that Wnt5a would suppress the growth of human EOC cells by antagonizing canonical Wnt/β-catenin signaling. To test our hypothesis, we examined the effect of Wnt5a reconstitution on expression of markers of active Wnt/β-catenin signaling in human EOC cells, namely the levels of "active" soluble β-catenin (21, 22, 32) and expression of β-catenin target genes such as *CCND1, c-MYC,* and *FOSL1* (12, 13). Indeed, we observed a decrease in soluble β-catenin in Wnt5a-reconstituted OVCAR5 cells compared with vector controls (Fig. 3E). Consistently, we also observed a significant decrease in the levels of β-catenin target genes in these cells, namely *CCND1*

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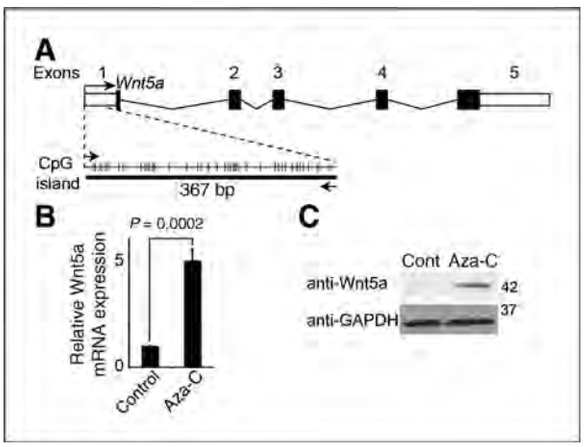

Figure 2. Promoter DNA CpG island hypermethylation contributes to Wnt5a downregulation in human EOC cells. A, schematic structure of the human *Wnt5a* gene transcript and its promoter CpG islands. Locations of exon 1 (open rectangle), CpG sites (vertical lines) and coding exons (filled rectangle), and the transcription start site (curved arrow) are indicated. Flat arrows indicate the positions of primers used for PCR amplification, and the size of PCR product is also indicated. B, PEO1 cells were treated with 5 μmol/L Aza-C for 4 days, and mRNA was isolated from control- and Aza-C–treated cells and examined for *Wnt5a* mRNA expression by qRT-PCR. Mean of 3 independent experiments with SD. C, same as (B) but examined for Wnt5a protein expression by immunoblotting.

($P = 0.0095$), *FOSL1* ($P = 0.0012$), and *c-MYC* ($P = 0.0286$; Fig. 3F). Similar effects of Wnt5a reconstitution on expression of markers of active Wnt/β-catenin signaling (such as decreased levels of soluble β-catenin) were also observed in PEO1 human EOC cells (Supplementary Fig. S2D), suggesting that this is not cell line specific. On the basis of these results, we conclude that Wnt5a suppresses the growth of human EOC cells by antagonizing canonical Wnt/β-catenin signaling in human EOC cells.

Wnt5a reconstitution drives cellular senescence in human EOC cells

Next, we sought to determine the cellular mechanism whereby Wnt5a inhibits the growth of human EOC cells. We have previously shown that suppression of canonical Wnt signaling promotes cellular senescence in primary human fibroblasts by activating the senescence-promoting histone repressor A (HIRA)/promyelocytic leukemia (PML) pathway (22). PML bodies are 20 to 30 dot-like structures in the nucleus of virtually all human cells. PML bodies are sites of poorly defined tumor suppressor activity and are disrupted in acute PML (33). PML has been implicated in regulating cellular senescence. For example, the foci number and size of PML bodies increase during senescence (33, 34) and inactivation of PML suppresses senescence (35). Activation of the HIRA/PML pathway is reflected by the recruitment of HIRA into PML bodies (36).

To determine whether Wnt5a reconstitution activates the HIRA/PML senescence pathway and induces senescence in EOC cells, we first sought to determine whether the HIRA/PML pathway is conserved in human ovarian epithelial cells. Ectopically expressing activated oncogenes (such as oncogenic *RAS*) is a standard approach for inducing senescence in a synchronized manner in primary human cells (1, 2, 19, 20). Indeed, ectopic expression of oncogenic *H-RAS*^{G12V} induced senescence of primary HOSE cells, as evident by an increase in SA-β-gal activity, a universal marker of cellular senescence (Supplementary Fig. S3A and B). Notably, the HIRA/PML pathway was activated during senescence of primary HOSE cells induced by oncogenic *RAS*, as evident by the relocalization of HIRA into PML bodies (Supplementary Fig. S3C and D). This result shows that the senescence-promoting HIRA/PML pathway is conserved in human ovarian epithelial cells. In addition, primary HOSE cells with HIRA foci displayed a marked decrease in BrdU incorporation, a marker of cell proliferation, compared with HIRA foci–negative cells (Supplementary Fig. S3E and F). This result is consistent with the idea that activation of the HIRA/PML pathway is directly correlated with senescence-associated cell growth arrest (37).

We next asked whether Wnt5a expression is regulated during natural senescence of primary HOSE cells. Indeed, we observed an increase in the levels of Wnt5a mRNA in senescent primary HOSE cells compared with young cells (Fig. 4A–C). In addition, we found that ectopic Wnt5a induces senescence of primary HOSE cells (Fig. 4D–F). Together, we conclude that Wnt5a plays a role in regulating senescence of primary HOSE cells.

As Wnt5a antagonizes canonical Wnt signaling in human EOC cells (Fig. 3E and F), we sought to determine whether Wnt5a restoration might activate the senescence-promoting HIRA/PML pathway and induce senescence in human EOC cells. Toward this goal, we examined the localization of HIRA in OVCAR5 EOC cells reconstituted with Wnt5a or vector control. Notably, there was a significant increase in the percentage of cells with HIRA localized to PML bodies in Wnt5a restored human EOC cells compared with controls (Fig. 5A and B; $P = 0.004$). In addition, we also observed an increase in the number and size of PML bodies in the Wnt5a restored OVCAR5 EOC cells (Fig. 5A), which are also established markers of cellular senescence (35, 38). Similarly, we observed activation of the HIRA/PML pathway by Wnt5a restoration in PEO1 human EOC cells (Supplementary Fig. S4A and B), suggesting that the observed effects are not cell line specific. Together, we conclude that Wnt5a reconstitution activates the HIRA/PML senescence pathway.

The p53 and pRB tumor suppressor pathways play a key role in regulating senescence (1). Thus, we sought to determine whether activation of the HIRA/PML pathway depends on the p53 and pRB pathways. Interestingly, p16^{INK4a}, the upstream repressor of pRB, is deleted in OVCAR5 human EOC cell line (39). In addition, the levels of total phosphorylated pRB were not decreased by Wnt5a, whereas the levels of cyclin D1/CKD4-mediated Serine 780 phosphorylation on pRB (pRBpS780) were decreased by Wnt5a (ref. 40; Fig. 5C and D). Furthermore, p53 is null in OVCAR5 cells (41). We conclude that activation of the HIRA/PML pathway is independent of the p53 and p16^{INK4a}.

We next sought to determine whether Wnt5a restoration induces SA-β-gal activity, a universal marker of cellular senescence (1). Indeed, SA-β-gal activity was notably induced by

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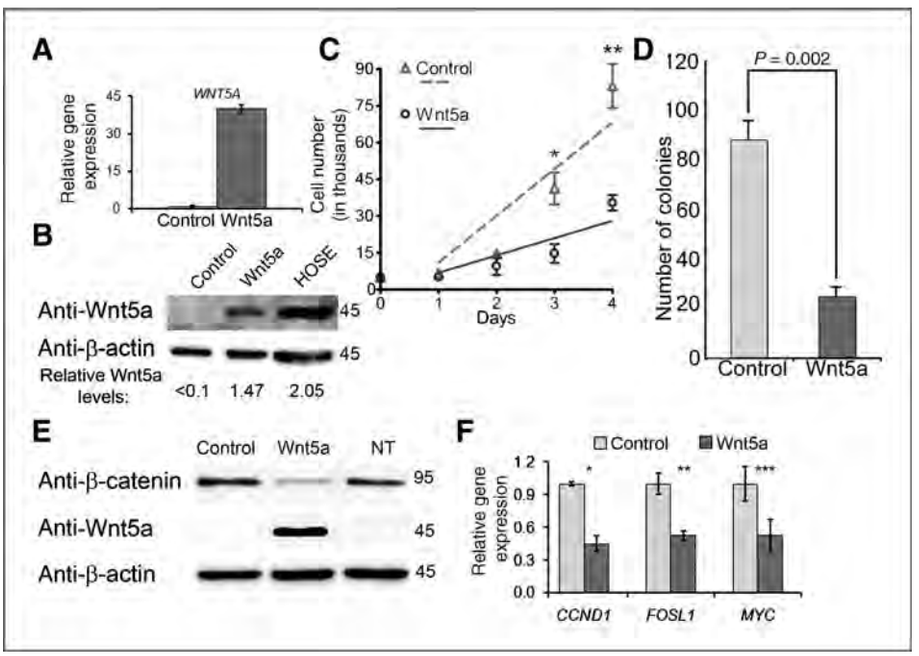

Figure 3. Wnt5a restoration inhibits the growth of human EOC cells by antagonizing canonical Wnt/β-catenin signaling. A, OVCAR5 cells were transduced with a control or Wnt5a-encoding puromycin-resistant retrovirus. The infected cells were drug-selected with 3 μg/mL puromycin. Expression of Wnt5a mRNA in drug-selected cells was determined by qRT-PCR. B, same as (A), but examined for expression of Wnt5a and β-actin in control or Wnt5a-infected OVCAR5 and primary HOSE cells by immunoblotting. Relative levels of Wnt5a expression was indicated on the basis of the densitometric analysis, using NIH ImageJ software. C, same as (A), but equal number (5,000) of drug-selected control (open triangles and dotted line) or Wnt5a-infected cells (open circles and solid line) were cultured on plastic plates for 4 days, and the number of cells was counted [control ± SD or Wnt5a ± SD ($n = 3$); Student's t test was used for calculating P value] at day 1 (6,666 ± 1,258 vs. 5,000 ± 1,000; $P = 0.1469$), day 2 (14,583 ± 954 vs. 9,583 ± 3,463; $P = 0.084$), day 3 (41,250 ± 6,538 vs. 14,750 ± 2,787; *, $P = 0.0038$), and day 4 (83,055 ± 8,978 vs. 35,416 ± 2,055; **, $P = 0.001$). Mean of 3 independent experiments with SD and linear regression. D, same as (C), but grown under anchorage-independent condition in soft agar. The number of colonies was counted 2 weeks after initial inoculation. Mean of 3 independent experiments with SD. E, same as (A), but examined for the levels of soluble β-catenin and β-actin expression by immunoblotting. NT, nontreated. F, same as (A), but examined for expression of indicated β-catenin target genes by qRT-PCR. Expression of β-2-microglobulin was used to normalize the expression of indicated genes. *, $P = 0.0095$; **, $P = 0.0012$; and ***, $P = 0.0286$ compared with controls.

Wnt5a reconstitution in both OVCAR5 and PEO1 human EOC cells compared with controls (Fig. 5E and F; Supplementary Fig. S4C and D, respectively). On the basis of these results, we concluded that Wnt5a restoration induced senescence of human EOC cells by activating the HIRA/PML senescence pathway.

Wnt5a inhibits the growth of human EOC cells *in vivo* by inducing cellular senescence

We next sought to determine whether Wnt5a would mediate growth inhibition and induce senescence *in vivo* in an orthotopic EOC model in immunocompromised mice. A luciferase gene was retrovirally transduced into control or Wnt5a-reconstituted OVCAR5 cells to monitor the cell growth *in vivo* via noninvasive imaging. These cells were injected unilaterally into the bursa sac covering the ovary in female immunocompromised mice ($n = 6$ for each of the groups; Supplementary Fig. S5). Tumor growth was monitored every 5 days starting at day 10 postinjection by measuring luciferase activity, and the growth of the tumor was followed for a total of 30 days (Fig. 6A). Wnt5a significantly suppressed the growth of xenografted OVCAR5 human EOC cells compared with controls (Fig. 6B; $P < 0.03$). Consistently, following general pathologic examination during surgical dissection at day 30, we observed that tumor sizes were notably smaller from mice injected with Wnt5a-reconstituted OVCAR5 cells compared with controls (data not shown). The expression of ectopic Wnt5a was confirmed by IHC staining in sections from dissected tumors (Fig. 6C).

We next sought to determine whether cell proliferation was suppressed by Wnt5a reconstitution in dissected tumors. Toward this goal, we examined the expression of Ki-67 by IHC. We observed, there was a significant decrease in the number of Ki-67–positive cells in tumors formed by Wnt5a-reconstituted OVCAR5 cells compared with controls (Fig. 6D and E). In addition, intensity of Ki-67 staining was also notably weaker in Ki-67–positive Wnt5A-reconstituted OVCAR5 cells than in control Ki-67–positive cells (Fig. 6D). On the basis of these results, we conclude that Wnt5a reconstitution inhibits the proliferation of human EOC cells *in vivo* in an orthotopic xenograft EOC model.

We next investigated whether the growth inhibition observed by Wnt5a reconstitution *in vivo* was due to induction of cellular senescence. Toward this goal, we examined the expression of SA-β-gal activity in fresh sections of dissected tumors formed by OVCAR5 cells reconstituted with Wnt5a or control cells. Indeed, we observed a significant increase in the number of cells positive for SA-β-gal activity in OVCAR5 cells reconstituted with Wnt5a compared with control tumors

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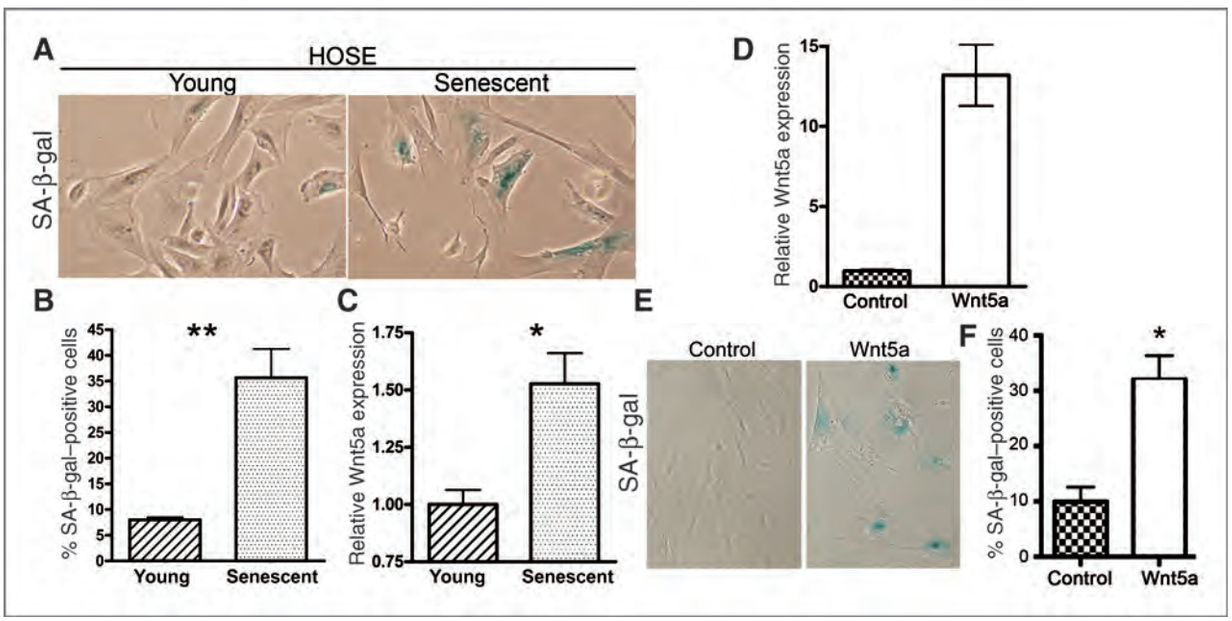

Figure 4. Wnt5a promotes senescence of primary HOSE cells. A, young proliferating primary HOSE cells were passaged to senescence (after 7 population doublings). Expression of SA-β-gal activity was measured in young and naturally senescent primary HOSE cells. B, same as (A). Quantitation of SA-β-gal–positive cells. **, $P < 0.001$. C, same as (A), but mRNA was isolated and examined for *Wnt5a* expression by qRT-PCR. Expression of B2M was used as a control. *, $P = 0.003$. D, young primary HOSE cells were transduced with retrovirus encoding human *Wnt5a* gene or a control. Expression of Wnt5a in indicated cells was determined by qRT-PCR. Expression of B2M was used as a control. E, same as (D), but stained for expression of SA-β-gal activity in drug-selected cells. F, quantitation of (E). Mean of 3 independent experiments with SD. *, $P < 0.05$.

(Fig. 6F and G; $P = 0.003$). Together, we conclude that Wnt5a reconstitution inhibits the growth of human EOC cells *in vivo* by inducing cellular senescence.

Discussion

Driving cancer cells to undergo cellular senescence has recently been proposed to be a novel mechanism to target for developing cancer therapeutics (1, 6). For example, pharmacologic inhibitor of *PTEN* drives senescence and, consequently, inhibits tumorigenesis *in vivo* in xenograft models of *PTEN* heterozygous prostate cancer cells (42, 43). Compared with apoptosis, therapeutics that drive cellular senescence are proposed to have less cytotoxic side effects (6), which makes prosenescence therapy attractive. Herein, we describe that restoration of Wnt5a signaling drives senescence of human EOC cells both *in vitro* and *in vivo* in an orthotopic mouse model of EOC (Figs. 5 and 6). Restoring gene expression by gene therapy has had limited success. Therefore, restoring Wnt5a signaling via exogenous ligand could prove to be an alternative approach. Interestingly, it has been previously reported that a Wnt5a-derived hexapeptide is sufficient to restore Wnt5a

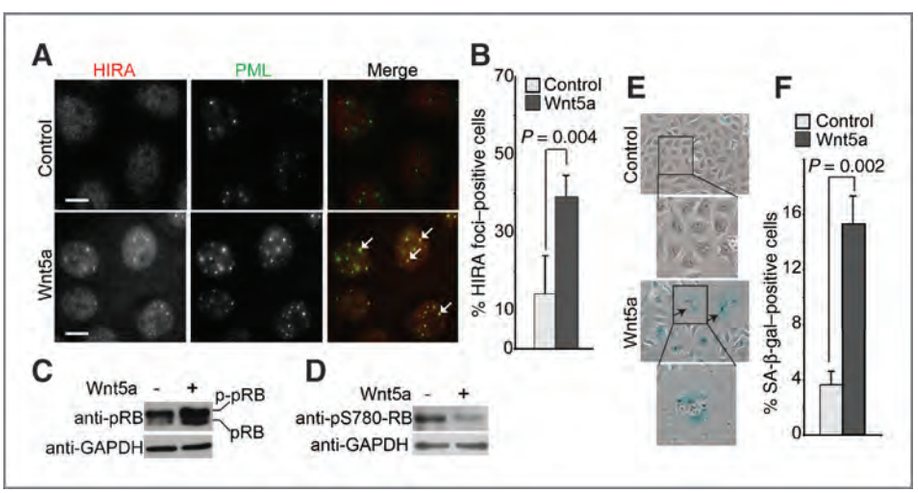

Figure 5. Wnt5a restoration triggers cellular senescence in human EOC cells. A, control and Wnt5a-expressing OVCAR5 EOC cells were stained with antibodies to HIRA and PML. Arrows point to examples of colocalized HIRA and PML bodies. Bar, 10 μm. B, quantitation of (A). A total of 200 cells from control and Wnt5a-expressing cells were examined for HIRA and PML colocalization. Mean of 3 independent experiments with SD. C, same as (A), but examined for pRB and GAPDH expression. D, same as (C), but examined for pRBpS780 and GAPDH expression. E, same as (A), but examined for SA-β-gal activity. F, quantitation of (E). Mean of 3 independent experiments with SD.

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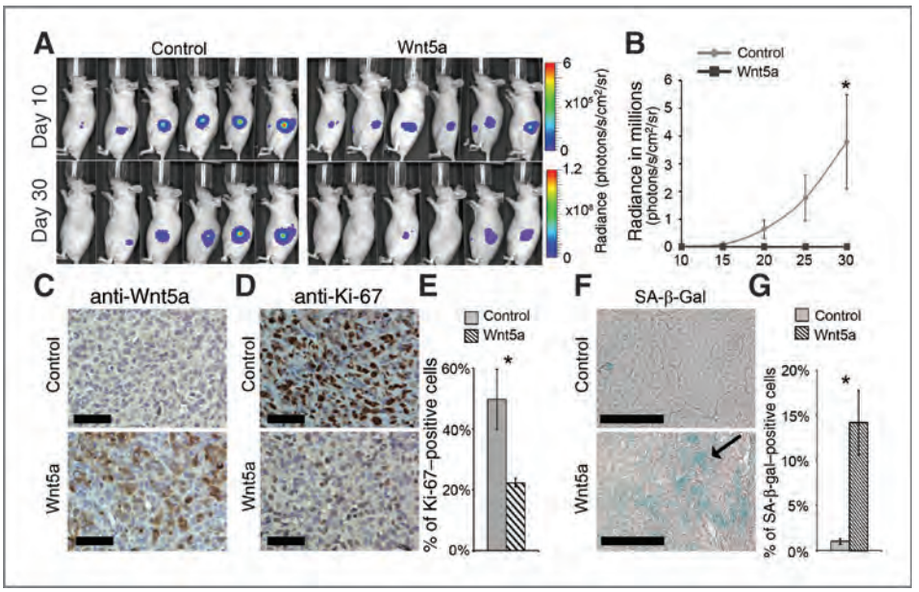

Figure 6. Wnt5a restoration inhibits tumor growth and promotes senescence of human EOC cells *in vivo*. A, OVCAR5 cells were transduced with luciferase-encoding hygromycin-resistant retrovirus together with a control or Wnt5a-encoding puromycin-resistant retrovirus. Drug-selected cells were unilaterally injected into the periovarian bursa sac of the female immunocompromised mice ($n = 6$ for each of the groups). The radiance of luciferase bioluminescence, an indicator of the rate for tumor growth, was measured every 5 days from day 10 until day 30 by using the IVIS imaging system. Shown are images taken at day 10 and day 30, respectively. B, quantitation of tumor growth from injected OVCAR5 cells expressing Wnt5a or control at indicated time points. *, $P = 0.038$ compared with controls. C, following tumor dissection, expression of Wnt5a in tumors formed by control or Wnt5a-expressing OVCAR5 EOC cells was determined by immunohistochemical staining against Wnt5a (magnification, 40×). Bar, 50 μm. D, same as (C), but examined for expression of Ki-67, a marker of cell proliferation (magnification, 40×). Bar, 50 μm. E, quantitation of (D). *, $P = 0.008$ compared with controls. F, expression of SA-β-gal activity was examined on sections of fresh-frozen tumors formed by OVCAR5 cells expressing control or Wnt5a (magnification, 40×). Bar, 100 μm. G, quantitation of (F). *, $P = 0.003$ compared with controls. Arrow points to an example of SA-β-gal positive cells.

signaling both *in vitro* and *in vivo* in xenograft models of breast cancer (44). It would be interesting to test whether the Wnt5a-derived hexapeptide will be sufficient to reconstitute Wnt5a signaling and drive senescence of EOC cells. Our data suggest that cellular senescence is a potential target for developing EOC therapeutics. In addition, these data imply that restoration of Wnt5a signaling represents a potential novel strategy to drive senescence of EOC cells.

This study is the first to show a role for Wnt5a in regulating senescence. We showed that Wnt5a activated the senescence-promoting HIRA/PML pathway in human EOC cells (Fig. 5A; Supplementary Fig. S4A). In primary human cells, activation of HIRA/PML pathway is sufficient to drive senescence by facilitating epigenetic silencing of proliferation-promoting genes (such as E2F target genes; ref. 19). Herein, we reported for the first time that the key HIRA/PML senescence pathway can be reactivated to drive senescence of human cancer cells. Further studies are warranted to elucidate the molecular basis by which Wnt5a restoration and activation of HIRA/PML pathway drive cellular senescence in human EOC cells.

Interestingly, senescence induced by Wnt5a restoration in human EOC cells was independent of both the p53 and $p16^{INK4a}$ tumor suppressors, which implies that EOC cells that lack p53 and $p16^{INK4a}$ retain the capacity to undergo senescence via HIRA/PML pathway through suppressing the canonical Wnt signaling. This is consistent with previous reports showing that cancer cells that lack p53 and pRB retain the capacity to undergo senescence when treated with anticancer agents or ionizing radiation (6). Notably, although the levels of total phosphorylated pRB were not decreased by Wnt5a, we observed a decrease in the levels of pRBpS780 that is mediated by cyclin D1/CDK4 (Fig. 5C and D). Future studies will determine whether the decrease in pRBpS780 levels plays a role in regulating senescence of human EOC cells.

Expression of Wnt5a is altered in many types of cancers (45). For example, in melanoma, Wnt5a overexpression correlates with cancer progression and a higher tumor stage (16). However, in colorectal and esophageal squamous cell carcinomas, Wnt5a has been described to be a tumor suppressor and was frequently silenced by promoter hypermethylation (16, 46). Consistently, we also observed *Wnt5a* promoter hypermethylation in a number of human EOC cell lines in which Wnt5a is downregulated (Fig. 2; Supplementary Table S1). This result is consistent with the idea that *Wnt5a* promoter hypermethylation contributes to Wnt5a downregulation in human EOC cells.

Wnt5a function is highly dependent on cellular context (45). For example, the cellular Wnt receptor/coreceptor context dictates the downstream signaling pathways upon the binding of Wnt5a, which include activating noncanonical Wnt signaling or antagonizing canonical Wnt/β-catenin signaling (47). These reports illustrate that Wnt5a expression and its resulting activity are cell type and context dependent. The Wnt receptor/coreceptor profile in EOC cells is currently unknown, and our future studies will elucidate the mechanism by which Wnt5a

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antagonizes Wnt/β-catenin signaling in human EOC cells. Regardless, our data show that Wnt5a downregulation is an independent predictor for overall survival in EOC patients. In contrast, 2 other studies showed that higher Wnt5a expression predicts poor survival in EOC patients (48, 49). The basis for this discrepancy remains to be elucidated. An explanation may be that our study included more cases than the other 2 studies (130 EOC cases in our study vs. 38 cases in the study by Badiglian and colleagues or 63 cases in the study by Peng and colleagues). It may also be due to the difference in the composition of type I and type II cases in this study compared with the other 2 studies. The vast majority of EOC cases in this study are of type II high-grade serous subtypes. Consistently, our data showed that there is a difference in Wnt5a expression between type I and type II EOC ($P = 0.005$; Table 1). Furthermore, it has been shown in microarray analysis that Wnt5a is expressed at lower levels in laser capture and microdissected high-grade serous EOC compared with normal primary HOSE cells (50).

In summary, the data reported here show that Wnt5a is often expressed at lower levels in human EOCs compared with either normal human ovarian surface epithelium or fallopian tube epithelium. A lower level of Wnt5a expression correlates with tumor stage and predicts shorter overall survival in EOC patients. Reconstitution of Wnt5a signaling inhibits the growth of human EOC cells both *in vitro* and *in vivo*. In addition, Wnt5a reconstitution suppresses the proliferation-promoting canonical Wnt/β-catenin signaling in human EOC cells. Significantly, Wnt5a reconstitution drives cellular senescence in human EOC cells and this correlates with activation of the senescence-promoting HIRA/PML pathway. Together, our data imply that reconstitution of Wnt5a signaling to drive senescence of human EOC cells is a potential novel strategy for developing EOC therapeutics.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Molecular Cancer Research

Enhancer of Zeste Homolog 2 Promotes the Proliferation and Invasion of Epithelial Ovarian Cancer Cells

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Cell Cycle, Cell Death, and Senescence

Molecular Cancer Research

Enhancer of Zeste Homolog 2 Promotes the Proliferation and Invasion of Epithelial Ovarian Cancer Cells

Hua Li[1], Qi Cai[2], Andrew K. Godwin[1], and Rugang Zhang[1,3]

Abstract

Enhancer of zeste homolog 2 (EZH2) is the catalytic subunit of the polycomb repressive complex 2 (PRC2) that includes noncatalytic subunits suppressor of zeste 12 (SUZ12) and embryonic ectoderm development (EED). When present in PRC2, EZH2 catalyzes trimethylation on lysine 27 residue of histone H3 (H3K27Me3), resulting in epigenetic silencing of gene expression. Here, we investigated the expression and function of EZH2 in epithelial ovarian cancer (EOC). When compared with primary human ovarian surface epithelial (pHOSE) cells, EZH2, SUZ12, and EED were expressed at higher levels in all 8 human EOC cell lines tested. Consistently, H3K27Me3 was also overexpressed in human EOC cell lines compared with pHOSE cells. EZH2 was significantly overexpressed in primary human EOCs ($n = 134$) when compared with normal ovarian surface epithelium ($n = 46$; $P < 0.001$). EZH2 expression positively correlated with expression of Ki67 ($P < 0.001$; a marker of cell proliferation) and tumor grade ($P = 0.034$) but not tumor stage ($P = 0.908$) in EOC. There was no correlation of EZH2 expression with overall ($P = 0.3$) or disease-free survival ($P = 0.2$) in high-grade serous histotype EOC patients ($n = 98$). Knockdown of EZH2 expression reduced the level of H3K27Me3 and suppressed the growth of human EOC cells both *in vitro* and *in vivo* in xenograft models. EZH2 knockdown induced apoptosis of human EOC cells. Finally, we showed that EZH2 knockdown suppressed the invasion of human EOC cells. Together, these data demonstrate that EZH2 is frequently overexpressed in human EOC cells and its overexpression promotes the proliferation and invasion of human EOC cells, suggesting that EZH2 is a potential target for developing EOC therapeutics. *Mol Cancer Res; 8(12); 1610–8. ©2010 AACR.*

Introduction

Enhancer of zeste homolog 2 (EZH2) is the catalytic subunit of polycomb repressive complex 2 (PRC2; refs. 1–4). In addition to EZH2, PRC2 also contains the noncatalytic subunits embryonic ectoderm development (EED) and suppressor of zeste 12 (SUZ12; ref. 5). PRC2 plays an important role in epigenetic gene silencing via methylation of lysine 27 residue of histone H3 (H3K27) and can add up to 3 methyl groups to the lysine side chain. EZH2 lacks enzyme activity on its own, and has to complex with EED and SUZ12 to attain robust histone methyltransferase activity (5, 6). The trimethylated form of H3K27 (H3K27Me3) is thought to be the main form that confers transcriptional silencing function (7–10).

EZH2 is overexpressed in several types of cancers (11–15) and is correlated with aggressiveness and poor prognosis in breast and prostate cancers (11–13). In breast epithelial cells, EZH2 overexpression causes anchorage-independent growth and increases cell invasiveness *in vitro* (11). In prostate cancer cells, inhibition of EZH2 blocked the growth of prostate cancer cells (13, 15). In addition, SUZ12 is also upregulated in certain types of cancer, including colon, breast, and liver (16–18).

More than 85% of ovarian cancers are of epithelial origin (19). Epithelial ovarian cancers (EOC) are classified into distinct histologic subtypes including serous, mucinous, endometrioid, and clear cell (19). The most common histology of EOC is serous (50%–60% of all EOCs), approximately, 75% of which is high-grade and 25% is low-grade (20–22). Less common histologies include endometrioid (25%), clear cell (4%), and mucinous (4%; ref. 20, 21). Recently, an alternative classification has gained traction, in which EOC is broadly divided into 2 types (22). Type I EOC includes endometrioid, mucinous, low-grade serous, and clear cell carcinomas, and type II EOC includes high-grade serous carcinomas (22). EOC remains the most

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Note: Supplementary data for this article are available at Molecular Cancer Research Online (http://mcr.aacrjournals.org/).

Authors' Contributions: H. Li performed all the experiments and drafted the manuscript. Q. Cai and A.K. Godwin reviewed and provided primary human ovarian carcinoma and normal ovary specimens. R. Zhang conceived the study, designed the experiments and wrote the manuscript.

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lethal gynecologic malignancy in the Western world (19). Thus, there is an urgent need to identify new targets for developing novel therapeutics for EOC. Although EZH2 is overexpressed in tumor-associated endothelial cells in invasive EOC (23) and regulates tumor angiogenesis in EOC (24), its role in pathogenesis of EOC remains poorly understood. Here, we examined the expression of the subunits of PRC2 and H3K27Me3 in human EOC cell lines. In addition, we determined EZH2 expression in primary human EOCs of different histologic subtypes by immunohistochemistry (IHC). Further, we investigated the effects of EZH2 knockdown by short hairpin RNA (shRNA) on H3K27Me3 expression, cell growth, and invasion of human EOC cells.

Material and Methods

Cell culture

Primary human ovarian surface epithelial (pHOSE) cells were isolated and cultured as previously described (25). The protocol was approved by Fox Chase Cancer Center (FCCC) institutional review board. Human EOC cell lines A1847, A2780, OVCAR3, OVCAR5, OVCAR10, PEO1, SKOV3, and UPN289 were kindly provided by Drs. Thomas Hamilton and Steve Williams at FCCC and were maintained in 1640 medium, supplemented with 10% FBS, 2 mmol/L of L-glutamine, penicillin (100 units/mL), and streptomycin (100 μg/mL).

shRNA, lentivirus packaging, and infection

The sense sequences of 2 individual shRNA EZH2 are: 5′-CCAACACAAGTCATCCCATTA-3′ and 5′-CGGAAATCTTAAACCAAGAAT-3′, respectively. Lentivirus packaging was performed using virapower system (Invitrogen) according to manufacturer's instruction. PEO1 and SKOV3 at 40% to 50% confluence were infected with lentivirus expressing shRNA to the human *EZH2* gene or vector control. The infected cells were drug-selected with 1 μg/mL (for PEO1) or 3 μg/mL (for SKOV3) of puromycin, respectively.

Human ovarian tissue microarrays

Tissue microarrays, including core samples from 134 primary human EOCs and 46 cases of normal ovary tissues were obtained from FCCC Biosample Repository Core Facility. Use of these human specimens was approved by the Institutional Review Board.

Immunohistochemical staining and scoring

The expression of EZH2 and Ki67 proteins was detected using avidin–biotin–peroxidase methods. Briefly, tissue sections were subjected to antigen retrieval by steaming in 0.01 mol/L of sodium citrate buffer (pH 6.0) for 30 minutes. After quenching endogenous peroxidase activity with 3% hydrogen peroxide and blocking nonspecific protein binding with 1% bovine serum albumin, sections were incubated overnight with primary monoclonal antibody (anti-EZH2: Millipore, 1:100; anti-Ki67: DAKO, 1:100) at 4°C, followed by biotinylated goat anti-mouse IgG (DAKO, 1:400) for 1 hour, detecting the antibody complexes with the labeled streptavidin-biotin system (DAKO), and visualizing them with the chromogen 3,3′-diaminobenzidine. Sections were lightly counterstained with hematoxylin. Tissues in which nuclei were stained for EZH2 or Ki67 protein were considered positive. Two 1-mm cores were examined in each specimen on the tissue microarray and cells were counted in at least 5 high-power fields, with approximately 200 cells analyzed per high-power field.

FACS, immunoflurescence staining, and Western blot analysis

FACS and indirect immunoflurescence (IF) staining were performed as described previously (26–28). The following antibodies were used for IF: rabbit anti-H3K27Me3 (Cell Signaling, 1:1,000), and rabbit anti-H3K9Me3 (Abcam, 1:500). The antibodies used for Western blotting were from indicated suppliers: mouse anti-EZH2 (Millipore; 1:2,500), rabbit anti-H3K27Me3 (Cell signaling, 1:1,000), rabbit anti-H3K9Me3 (Abcam, 1:2,000), mouse anti-histone H3 (Millipore, 1:10,000), mouse anti-GAPDH (Millipore, 1:10,000), rabbit anti-PARP p85 fragment (Promega, 1:1,000), rabbit anti-cleaved caspase 3 (Cell Signaling, 1:1,000), and rabbit anti-cleaved Lamin A (Cell signaling, 1:1,000).

Soft agar colony formation assay

A total of 1×10^4 cells per well were inoculated in a 6-well plate in 1.5 mL of RPMI 1640 medium supplemented with 10% FBS and 0.35% agar on a base layer of 1.5 mL of the same medium containing 0.6% agar. Three weeks after plating, the cells were stained with 1% crystal violet (Sigma) in PBS to visualize the colonies. Number of colonies that were larger than 50 μm (approximately 100 cells) in diameter in each well was counted.

Matrigel invasion assay

BD BioCoat Matrigel Invasion Chamber was used to measure cell invasion according to manufacturer's instruction. Cells (1×10^5 cells per well) suspended in 0.5 ml RPMI 1640 medium were added to the upper compartment of 24-well matrigel-coated or noncoated 8 μm membrane, and RPMI 1640 medium supplemented with 10% FBS was applied to the lower compartment. After incubating 22 hours at 37°C, 5% CO_2, the cells were fixed with 4% formaldehyde and stained with 1% crystal violet in PBS. The number of cells that migrated across control membrane or invaded through Matrigel-coated membrane was determined in 9 fields across the center and the periphery of the membrane.

Annexin V staining for detecting apoptotic cells

Phosphatidylserine externalization was detected using an Annexin V staining kit (Millipore) following manufacture's instruction. Annexin V positive cells were detected by

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Guava system and analyzed with Guava Nexin software Module (Millipore).

In vivo tumorigenicity assay

A total of 5×10^6 cells in PBS (pH 7.3) per mouse were injected subcutaneously into the flank of 6-week-old female nude athymic mice. The mice were sacrificed 4 weeks post-inoculation. Width and length of tumor size were measured and the tumor volume (mm^3) was calculated using the following formula: tumor volume (in mm^3) = length $\times$ width^2 $\times$ 0.52.

Statistical analysis

Quantitative data were expressed as mean $\pm$ SD, unless otherwise stated. analysis of variance (ANOVA) with Student's *t* test was used to identify significant differences in multiple comparisons. The χ^2 test was used to analyze the relationship between categorical variables. Overall survival was defined as the time elapsed from the date of diagnosis and the date of death from any cause or the date of last follow-up. Disease-free survival was defined as the time elapsed from the date of surgery and the date of the first recurrence. Kaplan–Meier survival plots were generated and comparisons made using the log-rank statistic. For all statistical analyses, the level of significance was set at 0.05.

Results

The catalytic and noncatalytic subunits of PRC2 complex and H3K27Me3 are expressed at higher levels in human EOC cell lines compared with pHOSE cells

Expression of EZH2, EED, and SUZ12 was examined by Western blotting in cultures of pHOSE cells isolated from 5 different individuals and 8 human EOC cell lines. When compared with pHOSE cells, EZH2, EED, and SUZ12 were expressed at higher levels in all human EOC cell lines tested (Fig. 1A). Consistently, the levels of H3K27Me3, the product of EZH2 histone H3 lysine 27 methyltransferase activity, was also increased in human EOC cell lines compared with pHOSE cells (Fig. 1A). On the basis of these results, we conclude that the catalytic and noncatalytic subunits of PRC2 and H3K27Me3 are expressed at higher levels in human EOC cell lines compared with pHOSE cells.

EZH2 is overexpressed in primary human EOCs and its expression positively correlates with expression of Ki67, a cell proliferation marker

We next sought to examine the expression of EZH2, the catalytic subunit of PRC complex, in 134 primary human EOCs and 46 normal ovary tissue specimens by IHC staining (Table 1; Fig. 1B; Supplementary Fig. S1). The specificity of the EZH2 antibody used for IHC staining was confirmed by the following (Supplementary Fig. S1A and B). First, a single band at right molecule weight (~95KD) was obtained in Western blotting of human EOC cell line SKOV3 using the EZH2 antibody, and this band was absent after expression of shRNA to the human *EZH2* gene (shEZH2) that effectively knocked down EZH2

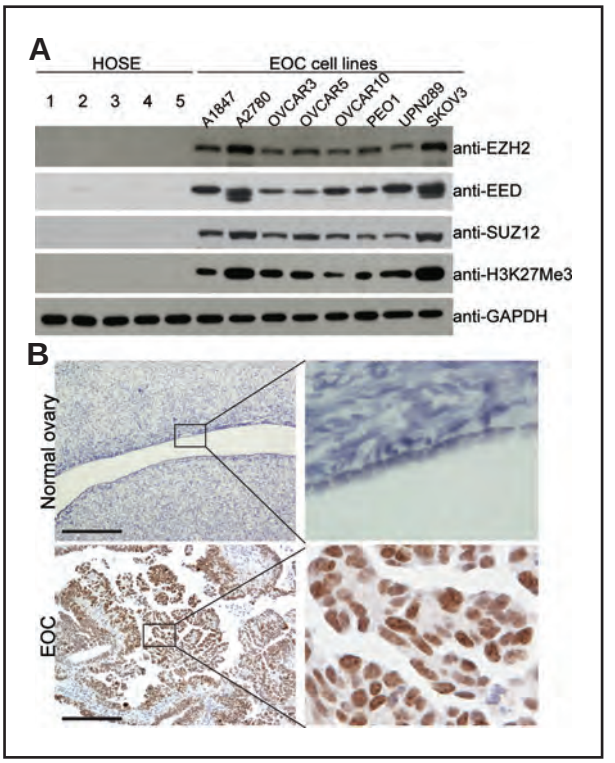

Figure 1. EZH2 is expressed at higher levels in human EOC cell lines and primary human EOCs compared with normal ovarian surface epithelium. A, expression of EZH2, EED, SUZ12, H3K27Me3, and GAPDH in 5 individual batches of pHOSE cell cultures and indicated human EOC cell lines was determined by Western blotting. B, examples of EZH2 immunohistochemical staining in normal ovary and primary human EOC (shown is an example of high-grade serous histotype EOC). Bar = 50 μm.

mRNA expression (Supplementary Fig. S1A and data not shown). In addition, EZH2 staining signal was lost when primary anti-EZH2 was replaced with an isotype-matched IgG control (Supplementary Fig. S1B). Importantly, the nuclei of human EOC cells were strongly stained by the anti-EZH2 antibody (Fig. 1B; Supplementary Fig. S1C). By contrast, ovarian surface epithelial cells were negative for EZH2 staining (Fig. 1B).

We scored expression of EZH2 as low (H-score $\leq$ 100) or high (H-score > 100) based on the histochemical score (29, 30), which considers both the intensity of staining and the percentage of positively stained cells. EZH2 expression in the surface epithelium of all 46 normal ovaries was scored as low (in fact, negative EZH2 staining). EZH2 was scored as low in 34% (46 of 134) and high in 66% (88 of 134) of primary EOCs tested, respectively. When compared with normal ovarian surface epithelium, EZH2 was expressed at significantly higher levels in primary human EOCs ($P < 0.001$). Because EZH2 has been implicated in promoting cell proliferation (12), we stained the same set of primary human EOC specimens with Ki67, a cell proliferation marker, and compared the expression of EZH2 and Ki67 expression in consecutive sections. There

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Table 1. Correlation between EZH2 expression and tumor cell proliferation (Ki67) or clinicopathologic variables

	EXH2 protein expression				
Patient characteristics	**Low (*n*)**	**High (*n*)**	**Total (*n*)**	**High (%)**	***P***
Age (23–85 y, mean 59.6 y)					
≤55	17	31	48	64.58	
>55	29	57	86	66.28	0.843
Laterality					
Left	12	25	37	67.57	
Right	7	18	25	72.00	
Bilaterality	22	39	61	63.93	0.764
Undetermined	5	6	11	54.55	
Histotype					
Epithelial ovarian cancer	46	88	134	65.67	
Type I	11	25	36	69.44	
Low-grade serous	2	0	2		
Endometrioid	5	8	13		
Mucinous	1	4	5		
Clear cell	2	6	8		
Others	1	7	8		
Type II	35	63	98	64.29	0.577[a]
High-grade serous	35	63	98	64.29	
Normal ovarian epithelium	46	0	46	0.00	<0.001[b]
Ki67					
0%–10%	27	2	29	6.90	
10%–40%	10	17	27	62.96	
40%–100%	9	69	78	88.46	<0.001
Tumor grade					
Well differentiation	6	3	9	33.33	
Moderate differentiation	10	12	22	54.55	
Poor differentiation	29	70	99	70.71	0.034
Undetermined	1	3	4	75.00	
Tumor stage					
Stage 1/2	12	21	33	63.64	
Stage 3/4	33	55	88	62.50	0.908
Undetermined	1	12	13	92.31	

[a]Compared with type I, $P = 0.577$.
[b]Compared with epithelial ovarian cancer, $P < 0.001$.

was a significant correlation between EZH2 expression and Ki67 expression ($P < 0.001$; Table 1). Together, we conclude that EZH2 is significantly overexpressed in primary human EOCs compared with normal ovarian surface epithelium and its expression correlates with a high proliferation index revealed by Ki67 staining.

EZH2 expression is positively correlated with tumor grade but not tumor stage, or overall or disease-free survival

We next sought to determine the correlation between EZH2 expression and clinical and pathologic features of human EOCs. There was a significantly positive correlation between EZH2 expression and tumor grade ($P = 0.034$; Table 1). However, EZH2 expression was not associated with tumor stage ($P = 0.908$; Table 1). Next, we sought to determine whether EZH2 expression correlates with prognosis of type II high-grade serous histotype EOC patients for which long-term follow-up data were available ($n = 98$). The difference in overall ($P = 0.3$) or disease-free ($P = 0.2$) survival between low EZH2 expression group ($n = 35$) and high EZH2 expression group ($n = 63$) was not significant (Fig. 2).

EZH2 knockdown inhibits the growth of human EOC cells *in vitro* and *in vivo*

Because EZH2 expression positively correlates with Ki67 expression (Table 1), we sought to determine the effects of EZH2 knockdown on proliferation of human EOC cells. To

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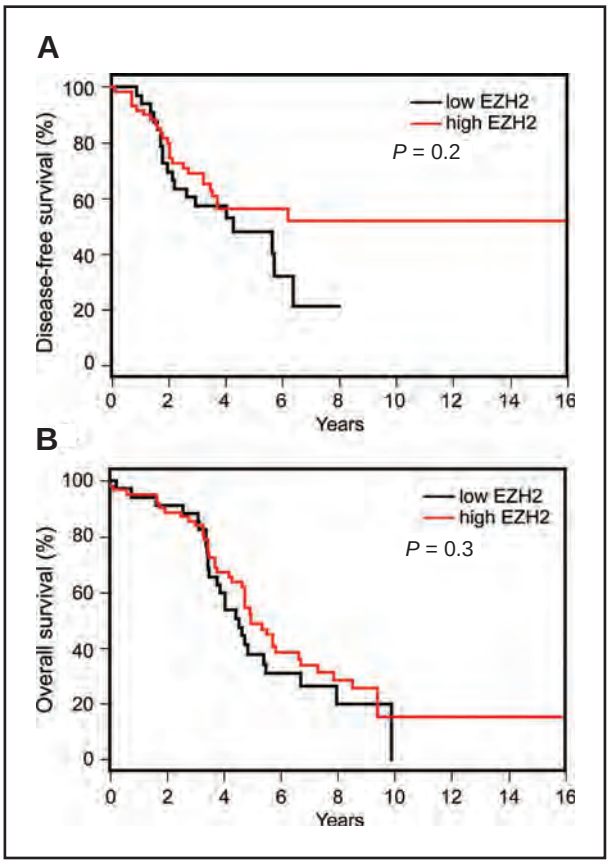

Figure 2. EZH2 expression does not correlate with disease-free or overall survival in high-grade serous histotype EOC patients. The univariate disease-free (A) and overall survival (B) curves (Kaplan–Meier method) for high-grade serous histotype EOC patients with low or high EZH2 protein levels as assessed by immunohistochemistry analysis.

knockdown EZH2 expression in SKOV3 cells, we developed 2 individual lentivirus encoded shEZH2. The knockdown efficacy of shEZH2 in SKOV3 cells was confirmed by Western blotting (Fig. 3A). Consistently, the level of H3K27Me3 level was significantly reduced by shEZH2 expression in SKOV3 cells as determined by both Western blotting and IF staining (Fig. 3B and C). As a negative control, shEZH2 expression has no effects on the level of trimethylated lysine 9 histone H3 (H3K9Me3) that is generated by histone methyltransferase Suv39H (ref. 31; Fig. 3B and C). Compared with controls, EZH2 knockdown significantly reduced both anchorage-dependent and -independent growth in soft agar in SKOV3 cells ($P < 0.001$; Fig. 3D and E). The degree of growth inhibition by shEZH2 correlated with the level of EZH2 knockdown in SKOV3 cells by 2 individual shEZH2 (Fig. 3), suggesting that the observed growth inhibition by shEZH2 was not because of off-target effects. In addition, EZH2 knockdown in PEO1 cells has same effects on the expression of H3K27Me3 and also suppressed both anchorage-dependent and -independent cell growth (Supplementary Fig. S2), suggesting that the observed growth inhibition is not cell line specific.

We next sought to determine the effects of EZH2 knockdown on the growth of SKOV3 cells *in vivo* in immunocompromised nude mice. Control and shEZH2 expressing SKOV3 cells were injected subcutaneously into nude mice with 5×10^6 cells per mice and 5 mice in each group. Four weeks after injection, the sizes of xenografted tumors were compared between control and shEZH2 expressing cells (Fig. 4A and B). EZH2 knockdown by shEZH2 in the xenograft tumors was confirmed by IHC staining (Fig. 4C). shEZH2 expression significantly inhibited the growth of xenografted SKOV3 cells (Fig. 4A and B).

EZH2 knockdown inhibits the invasion of human EOC cells

EZH2 has been implicated in regulating cell invasion in several types of cancer cells (11, 15, 32, 33). Thus, we sought to determine the effects of EZH2 knockdown on invasion of human EOC cells. Toward this goal, control and shEZH2 expressing SKOV3 cells were tested for their ability to migrate through uncoated control membrane or invade through matrigel-coated membrane. Compared with controls, EZH2 knockdown significantly inhibited the invasion of SKOV3 cells as revealed by a decreased invasion index that is calculated as the ratio between the number of cells invaded through matrigel-coated membrane and the number of cells migrated through control membrane (Fig. 5). Inhibition of invasion was observed by 2 individual shEZH2 in SKOV3 cells (Fig. 5). In addition, the degree of invasion inhibition correlated with the degree of EZH2 knockdown (Figs. 3 and 5), suggesting that this is not because of off-targets effects. On the basis of these results, we conclude that EZH2 knockdown inhibits the invasion of human EOC cells.

EZH2 knockdown triggers apoptosis in human EOC cells

We next sought to determine the mechanisms by which EZH2 knockdown inhibits the growth of human EOC cells. DNA content analysis determined by FACS showed that there was no statistical difference in cell-cycle distribution between control and shEZH2 expressing cells (Supplementary Fig. S3). We next examined the markers of apoptosis in control and shEZH2 expressing SKOV3 cells. When compared with controls, markers of apoptosis were significantly induced by shEZH2 expression (Fig. 6). Those apoptotic markers include increased percentage of cells at sub-G1 phase as measured by FACS analysis (Fig. 6A), increased percentage of Annexin V positively stained cells as measured by Guava Nexin assay (Fig. 6B), upregulation of cleaved Lamin A, PARP p85, and caspase 3 (Fig. 6C; ref. 34). Together, we conclude that EZH2 knockdown induces apoptosis of human EOC cells.

Discussion

Consistent with our findings, EZH2 mRNA expression was upregulated 2-fold or more in more than 80% of high-grade serous human EOC specimens in the newly released

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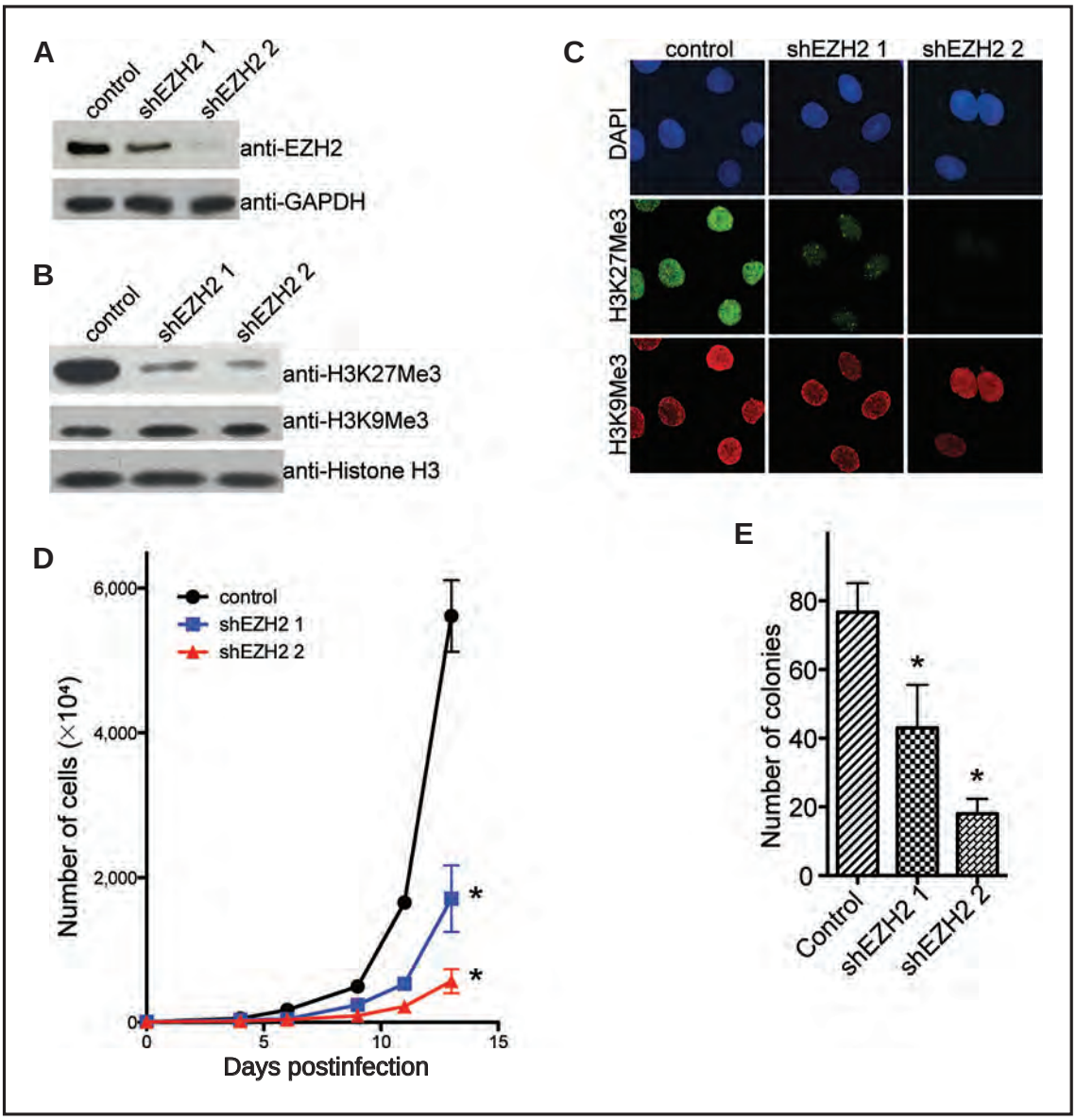

Figure 3. EZH2 knockdown inhibits the growth of SKOV3 cells *in vitro*. A, SKOV3 cells were infected with 2 individual lentivirus encoded shEZH2 or control. Drug-selected cells were examined for expression of EZH2 and GAPDH by Western blotting. B, same as A, but examined for expression of H3K27Me3 and H3K9Me3 by Western blotting. C, same as A, but examined for expression of H3K27Me3 and H3K9Me3 by immunofluorescence staining. DAPI counterstaining was used to visualize the cell nuclei. D, same as A, but equal number of drug-selected cells were seeded and counted at indicated time points. Mean of 3 independent experiments with SD. *, $P < 0.001$. E, same as A, 1×10^4 cells were seeded in soft agar and the number of colonies were counted after 3 weeks of culture. Mean of 3 independent experiments with SD. *, $P < 0.001$.

the Cancer Genomics Atlas (TCGA) serous ovarian cystoadenocarcinoma gene expression database (http//cancergenome.nih.gov/). *EZH2* gene is located at chromosome 7q36.1. Gene amplification contributes to EZH2 overexpression in several types of cancer (14, 35). However, TCGA gene copy-number analysis indicates that *EZH2* gene amplification occurs only in a very small percentage of EOCs (<10% specimens show >4 copy of EZH2 gene; http//cancergenome.nih.gov/), suggesting that gene amplification is not a major mechanism that leads to EZH2 upregulation in human EOCs. EZH2 is an E2F target gene (35). A very recent study showed that VEGF stimulates EZH2 expression in human EOC cells via E2F family members, E2F1 and E2F3 (24). However, VEGF only stimulates the expression of EZH2 mRNA up to 3-fold (24), which is far below the level of increase in EZH2 mRNA or protein in human EOC cells compared with cultured pHOSE cells (Fig. 1 and data not shown), suggesting additional mechanisms contribute to EZH2 upregulation in human EOC cells. In the future, we will elucidate

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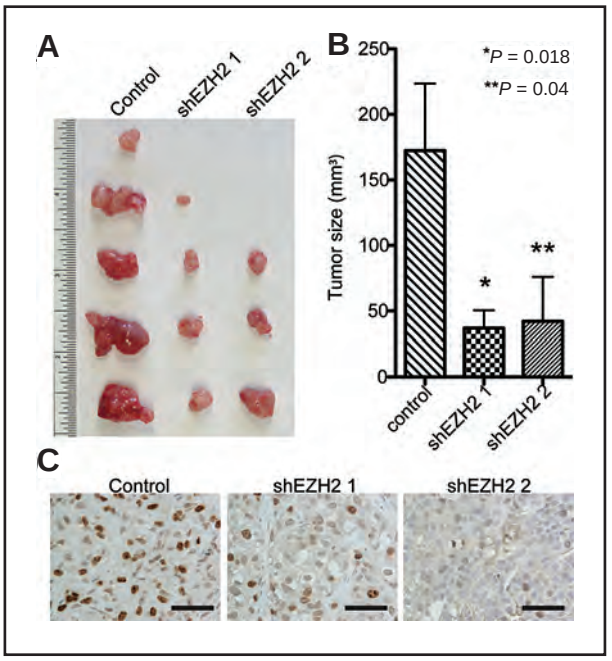

Figure 4. EZH2 knockdown suppresses the growth of SKOV3 cells *in vivo* in immunocompromised mice. A, 5×10^6 control or shEZH2 expressing SKOV3 cells were injected subcutaneously into immunocompromised nude mice ($n = 5$). Four weeks postinjection, tumors were removed from mice. B, quantitation of A, the size of tumors was measured. Mean of tumor sizes with SEM. C, xenografted tumors formed by control or shEZH2 expressing SKOV3 cells were sectioned and stained for EZH2 expression. Bar = 50 μm.

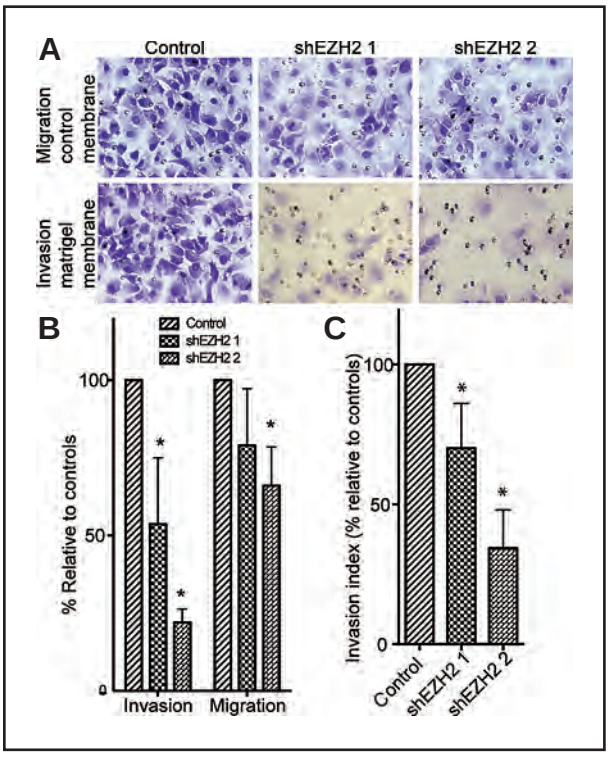

Figure 5. EZH2 knockdown suppresses the invasion of SKOV3 cells. A, equal number of control and shEZH2 expressing SKOV3 cells were assayed for migration through uncoated control membrane or invasion through matrigel-coated membrane. The cells migrated through control membrane or invaded through matrigel-coated membrane were stained with 1% crystal violet in PBS. B, quantitation of A. Relative percentage of shEZH2 expressing cells migrated through control membrane or invaded through matrigel-coated membrane compared with controls was indicated. Mean of 3 independent experiments with SD. *, $P < 0.05$. C, invasion index of shEZH2 expressing SKOV3 cells compared with controls. Invasion index is the ratio between cells invaded through matrigel-coated membrane and cells migrated through control membrane. Mean of 3 independent experiments with SD. *, $P < 0.05$.

additional mechanisms that contribute to EZH2 upregulation in human EOCs.

EZH2 has been demonstrated as a prognostic marker for breast and prostate cancers and positively correlates with disease-free survival and overall survival in those patient populations (11–13). In addition, EZH2 overexpression correlates with more advanced disease stages of breast and prostate cancers (11, 13). However, EZH2 expression was not a prognostic marker in other types of cancers including renal clear cell carcinoma and hepatocellular carcinomas (33, 36). We showed that there was no significant correlation between EZH2 expression and disease-free or overall survival in high-grade serous EOC patients (Fig. 2). Consistent with our findings, low expression of H3K27Me3 has been demonstrated to be a poor prognostic marker in EOC (37). In contrast, a very recent study showed that EZH2 expression in either EOC cells or ovarian tumor vasculature is predictive of poor clinical outcome (24). The basis for the discrepancy between our study and that of Lu et al.'s is unclear. A possible reason may be that we correlated EZH2 expression with overall or disease-free survival only in high-grade serous histotype EOCs (Fig. 2), whereas the study by Lu et al. includes additional histotypes of EOCs (24).

We showed that EZH2 expression positively correlated with Ki67 expression in EOCs (Table 1). There are conflicting results regarding the prognostic value of Ki67 in ovarian carcinoma (38–42). A recent study by Kobel et al. (43) suggests that differences in Ki67 index among different subtypes of EOCs confound the Ki67 survival analysis because nearly all high-grade serous EOCs have a high Ki67 index. In analysis of each individual subtypes, Ki67 is no longer a prognostic marker. Consistent with this, although EZH2 correlates with Ki67 expression (Table 1), EZH2 expression was not a prognostic indicator for either overall or disease-free survival in the tested high-grade serous histotype EOC patients (Fig. 2).

Interestingly, when compared with normal ovarian surface epithelium, EZH2 expression is significantly upregulated (up to 23-fold) in ovarian epithelial inclusion cysts (44), which are thought to be the precursor lesion of a subset of EOC (45). This suggests that EZH2 overexpression is an early event during EOC development. Although ovarian surface epithelium is thought to be the cell origin of EOC (46), there are still several histopathology-based theories that differ in their explanations about the origins of EOC (46–48). Notably, recent evidence suggests that a proportion of high-grade serous EOC may arise from distal fallopian tube

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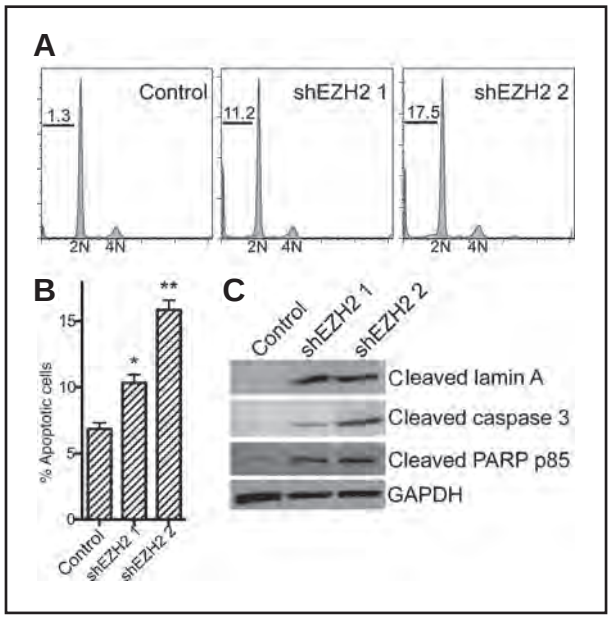

Figure 6. EZH2 knockdown induces apoptosis of SKOV3 cells. A, control and shEZH2 expressing SKOV3 cells were examined for cell-cycle distribution by FACS. The percentage of sub-G1 cells was indicated. B, control and shEZH2 expressing cells were stained for Annexin V, a cell surface marker of apoptosis. Annexin V–positive cells were measured by Guava Nexin assay. Mean of 3 independent experiments with SD. *, P, 0.019 and **, $P < 0.001$ compared with controls. C, same as B, but examined for expression of cleaved Lamin A, PARP p85, and caspase 3, all markers of apoptosis in control and shEZH2 expressing cells.

(48). Therefore, it will be interesting to examine EZH2 expression in normal fallopian tube epithelium.

Multiple genes have been implicated in EZH2 inhibition–induced apoptosis. For example, FBXO32 contributes to EZH2 inhibition induced–apoptosis in breast cancer cells (45), and Bim expression has been demonstrated to mediate EZH2 inhibition–induced apoptosis in prostate cancer cells (49). Likewise, E-cadherin, DAB2IP, and SLIT2 have all been implicated in mediating increased invasiveness conferred by high levels of EZH2 expression (32, 50–52). Further studies are warranted to delineate the molecular mechanisms by which EZH2 overexpression promotes proliferation and invasion of human EOC cells.

In summary, the data reported here show that EZH2 is overexpressed in approximately 66% of primary human EOCs and its overexpression correlates with a high proliferation index and tumor grade in EOCs. Knockdown of EZH2 inhibits the growth of human EOC cells *in vitro* and *in vivo*. EZH2 knockdown induces apoptosis of human EOC cells. In addition, EZH2 knockdown suppresses the invasion of human EOC cells. Further, inhibition of the growth and invasion of human EOC cells induced by EZH2 knockdown correlates with a decrease in the levels of H3K27Me3, suggesting that EZH2 histone methyltransferase activity is critical for its function in human EOC cells. Together, our data imply that EZH2 is a potential target for developing epigenetic modifying therapeutics for EOC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were declared.

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AACR Annual Meeting Abstract

Wnt5a-dependent induction of senescence suppresses epithelial ovarian cancer.

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Epithelial Ovarian Cancer (EOC) remains the most lethal gynecological malignancy in US and is the fifth leading cause of cancer deaths among American women. Thus, there is an urgent need to understand the etiology of EOC to develop novel therapies for this disease. Here, we demonstrated that a non-canonical Wnt ligand, Wnt5a, is expressed at significantly lower levels in human EOC cell lines and in primary human EOC compared with either normal ovarian surface epithelial cells or fallopian tube epithelial cells. Importantly, expression of Wnt5a in primary human EOC inversely correlates with tumor stage but not tumor grade. Interestingly, Wnt5a expression is significantly lower in Type II high-grade serous EOC compared to Type I EOC that includes low-grade serous, mucinous, clear cell and endometrioid subtypes of EOC. In addition, we discovered that hypermethylation of promoter CpG island contributes to Wnt5a downregulation in human EOC cells. Significantly, restoration of Wnt5a expression in human EOC cells promoted senescence of EOC cells and resulted in a dramatic decrease in cell proliferation both *in vitro* and *in vivo* in an orthotopic model of EOC in the ovary of SCID mice. Mechanistically, Wnt5a inhibited canonical Wnt/β-catenin signaling and resulted in the activation of senescence-promoting histone repressor A/PML pathway. In summary, we show that Wnt5a is often expressed at lower levels in primary human EOC and Wnt5a expression suppresses the growth of EOC cells by triggering senescence through antagonizing canonical Wnt signaling. These results also suggest that loss of Wnt5a expression is a putative marker of EOC and that non-canonical Wnt signaling is a potent target for developing novel EOC therapeutics.